Caldesmon−Actin−Tropomyosin Contains Two Types of Binding Sites for Myosin S1†

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ABSTRACT: Caldesmon inhibits the activation of myosin ATPase activity by actin−tropomyosin. Caldesmon also inhibits the binding of myosin to actin. There is disagreement as to the degree to which competitive displacement of myosin subfragment binding to actin is responsible for the inhibition of ATPase activity. We have examined the possibility that one or more molecules of S1 may bind to actin−tropomyosin−caldesmon without having the normal actin activation of ATPase activity. The effect of caldesmon on the binding and ATPase activity of S1 was measured at several initial levels of saturation of S1 to determine if a fraction of the bound S1 was resistant to displacement by caldesmon. In the case of both unmodified S1 and ρPDM-modified S1, most, but not all, of the S1 was displaced by caldesmon. The results are consistent with a single molecule of S1 binding with low affinity for each seven actin monomers that are fully saturated with caldesmon and tropomyosin. This single S1 is not necessarily bound directly to actin but may be attached to the NH_2-terminal region of caldesmon.

Caldesmon is an actin- and calmodulin-binding protein which inhibits actomyosin superprecipitation (*1*) and the actin-activated ATPase of myosin (*2*−*6*). The region of caldesmon which contains the actin binding site and is responsible for inhibition of ATPase activity is the carboxyl terminal domain between residues 440 and 756 (*7*, *8*), although other regions of actin binding have been proposed (*9*, *10*). Since caldesmon may be part of a regulatory system that is not present in skeletal or cardiac muscle, it is of interest to determine the mechanism by which caldesmon inhibits ATPase activity and contraction.

The hydrolysis of ATP by myosin and its stimulation by actin is a complex process. The most basic question regarding the effect of a regulatory protein on this process is whether that protein alters the binding of myosin to actin, the distribution of actin between conformationally active and inactive states, or whether it alters the transition between different myosin−nucleotide intermediate states (*11*). Several studies have shown that caldesmon inhibits the binding of different myosin nucleotide complexes to actin (*12*−*20*). The addition of caldesmon to single psoas fibers causes a decrease in the active force and a coincident decrease in myosin cross-bridge binding to actin (*21*−*23*). Caldesmon also inhibits actin−myosin interactions in ghost fibers (*17*), and inhibits in vitro motility (*24*). These results suggest that caldesmon and myosin may be competing for a common binding site on actin and that this competition results in an inhibition of actomyosin ATPase. The skeletal fiber studies are particularly informative since a large fraction of the S1[1] is displaced under conditions where the geometry in the fiber increases the likelihood of binding of myosin to actin. Therefore, there is good evidence that caldesmon functions as a competitive inhibitor of the binding of myosin to actin.

Solution studies have been done using myosin subfragments prepared from both smooth and striated muscle. While caldesmon has similar effects on the ATPase activity of smooth muscle HMM and skeletal muscle S1, the apparent effects on binding are different. While caldesmon caused a parallel decrease in the amount of skeletal S1−ATP which migrated with actin in an ultracentrifuge, caldesmon caused an increase in the amount of smooth HMM which comigrated with actin (*6*, *25*). We proposed that in these sedimentation experiments, the smooth HMM was not bound to actin but was attached to the caldesmon (*25*). We (*25*, *26*) and others (*27*) demonstrated that caldesmon does bind to myosin. Various myosin subfragments bound to caldesmon with affinities in decreasing order: smooth HMM > skeletal HMM > smooth S1 > skeletal S1. The myosin binding region of caldesmon was shown to be at the NH_2-terminus, distinct from the actin binding region (*28*). Removal of this region from caldesmon resulted in inhibition of HMM association with actin in an ultracentrifuge in the same manner as had been observed earlier with skeletal S1 (*20*, *28*).

There remain indications that factors other than displacement of myosin subfragments from actin may be involved in the inhibition of ATPase activity. When binding experiments were conducted at much higher concentrations of skeletal S1 than had been used previously (*12*, *25*, *28*)

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[1] Abbreviations: S1, myosin subfragment 1; ρPDM, *N*,*N*′-ρ-phenylenedimaleimide.

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complete inhibition of ATPase activity occurred at concentrations of either caldesmon or the carboxyl-terminal 20 kDa fragment which had little effect on the degree of association of S1 with actin (*29*, *30*).

Because a single caldesmon molecule binds to several actin monomers, it is possible that caldesmon inhibits the binding of S1 to only a subset of the actin monomers under its influence. We have considered this possibility earlier (*14*). It is also possible that one molecule of S1 may bind to the NH_2-terminal region of a caldesmon molecule that is bound to actin while all of the S1 binding sites of the actin are blocked (*25*). Both of these possibilities would result in a fraction of S1 that can remain bound to a caldesmon−actin−tropomyosin complex. We have attempted to estimate the fraction of S1 that can bind to the caldesmon−actin−tropomyosin complex under optimal conditions. The results indicate that caldesmon is able to displace bound S1 from most of the actin monomers. A small amount of S1, equivalent to one bound S1 per set of seven actin monomers, does remain bound to the caldesmon−actin−tropomyosin complex; this residual S1 may be bound to caldesmon directly.

EXPERIMENTAL PROCEDURES

Protein Preparation. Skeletal actin was isolated from rabbit back and leg muscle by a modification of the method of Spudich and Watt (*31*, *32*). Skeletal muscle myosin was isolated from the back and leg muscles of rabbits by standard procedures (*33*). Skeletal muscle S1 was made by the method of Weeds and Taylor (*34*). Smooth muscle tropomyosin was prepared from turkey gizzards (*35*). Caldesmon was purified from turkey gizzards either by a modification of the heat-treatment method of Bretscher (*35*) or by a method which avoids heat treatment as described previously by Velaz et al. (*36*). The 20 kDa caldesmon fragment was prepared as described previously (*15*).

S1 was modified with ρPDM at 0 °C in the presence of ADP as described by Wells and Yount (*37*). The contaminant unmodified S1 was removed by sedimentation with actin as described previously (*38*). For binding studies, S1 was modified with [^{14}C]ρPDM (specific activity of 7.2×10^3 cpm/nmol). [^{14}C]ρPDM was synthesized as described by Wells and Yount (*37*) and was purified by sublimation. ρPDM from Aldrich was sublimated before use.

The concentrations of skeletal myosin and skeletal actin were determined by absorbance at 280 nm, whereas the concentrations of smooth tropomyosin and caldesmon were determined by the Lowry assay (*39*) with bovine serum albumin as a standard. The molecular masses used for calculation of protein concentrations were 120 000 for S1, 42 000 for actin, 68 000 for tropomyosin, 87 000 for caldesmon, and 20 000 for the actin binding fragment of caldesmon. The purity of all proteins was verified by SDS−polyacrylamide gel electrophoresis.

Binding Studies. Prior to doing binding assays, S1 was clarified by centrifugation at 45 000 rpm for 30 min in a 50 Ti rotor (Beckman). The binding of S1 to actin−tropomyosin in the presence of ATP was measured by sedimenting the acto−S1 in an ultracentrifuge and determining the free S1 concentration by an NH_4^+/EDTA-ATPase assay as described earlier (*40*, *12*). At the highest concentrations of S1 used in this study, the ATP was sometimes depleted during the sedimentation and the fraction of S1 bound increased dramatically. The restriction to use low ionic strength required the use of a less active S1 preparation rather than more ATP or an ATP-regenerating system to avoid ATP depletion. Therefore, some experiments were done using S1 modified with [^{14}C]ρPDM to a specific activity of $(7-8) \times 10^3$ cpm/nmole. The utility of this modification for studying S1−ATP-like states has been shown previously (*38*, *41*). The fraction of bound S1 was calculated by the difference in radioactivity of the supernatant before and after centrifugation (*38*). All binding data were corrected for the fraction of nonspecific sedimentation of S1 in the absence of any actin. The conditions for the binding experiments are given in the figure legends.

ATPase Assays. The ATPase rates of S1 and ρPDM−S1 were measured by the rate of liberation of [^{32}P]phosphate from γ-^{32}P-labeled ATP (*40*). Three to five time points were taken for each determination to ensure that the reaction was linear.

RESULTS

Our earlier studies of the effect of caldesmon on S1−ATP binding to actin−tropomyosin were done using a very low S1 concentration ($\approx$0.1 μM) so that the ATP would not be depleted during the binding assay (*12*, *25*, *26*). The depletion of ATP would lead to rigor binding which is more resistant to the effects of caldesmon (*12*, *25*). Another laboratory reported optimal conditions for detecting the binding of S1 to caldesmon−actin−tropomyosin (*29*, *30*). These conditions included a 60-fold higher S1 concentration than that used in our studies. Under these conditions, S1 was not displaced by caldesmon and yet the ATPase activity was not activated by actin (*29*, *30*). This was interpreted as evidence that caldesmon does not displace S1−ATP in the presence of tropomyosin but rather inhibits the actin activation of ATP hydrolysis. Our first aim was to examine the binding of S1 to caldesmon−actin−tropomyosin under conditions which favor this association.

In our hands, utilizing such high S1 concentrations at 25 °C resulted in total hydrolysis of ATP during the binding reaction. Lowering the temperature to 10 °C resulted in 25% of the ATP remaining at the end of the reaction (determined by including [^{32}P]ATP in the binding assay). This amount of ATP was sufficient to ensure saturation of S1 with ATP. Figure 1 shows the effect of caldesmon on the binding of 6 μM S1 to actin at 10 °C. As was the case with lower S1 concentrations (for example, ref 12, Figures 1 and 6; ref 26, Figure 1), the ATPase activity decreased with increasing caldesmon concentrations. However, in contrast to experiments done at very low S1 concentrations, there was little effect of caldesmon on the amount of S1−ATP which comigrated with actin in the ultracentrifuge. At all caldesmon concentrations, the fraction of actin−tropomyosin with bound S1 was 0.04. That is, one molecule of S1 comigrated with each 25 actin monomers.

There are several reasons why caldesmon might inhibit the binding of S1 to actin−tropomyosin at low concentrations of free S1 but not at high concentrations of free S1. First, S1−ATP may bind with low affinity to the caldesmon−actin−tropomyosin complex, and increasing the S1 concen-

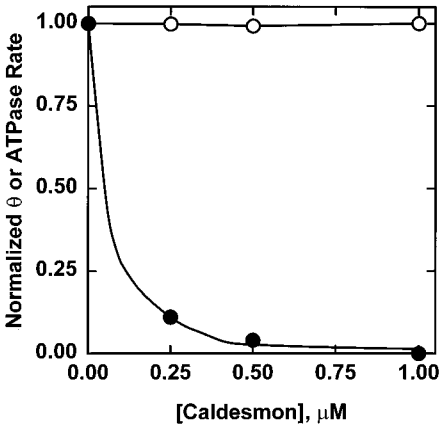

FIGURE 1: Effect of caldesmon on the ATPase activity (closed circles) and the binding (open circles) of S1 to actin−tropomyosin at $\mu = 40$ mM, 10 °C, 6 μM S1, 4 μM actin, and 0.86 μM smooth muscle tropomyosin. The final buffer conditions were 4 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 5 mM potassium propionate, 0.5 mM EGTA, and 5 mm Mg-ATP. Theta is the ratio of the S1 bound to the total actin concentration and was 0.038 in the absence of caldesmon. The ATPase rate (μM ATP hydrolyzed per second per μM S1) in the absence of caldesmon was 0.28 s^{-1}.

tration would increase the amount of bound S1−ATP. This low affinity binding could occur so that S1−ATP binds to every actin monomer that is covered by caldesmon. S1−ATP might also bind to a subpopulation of the actin monomers covered by caldesmon. It is also possible that S1−ATP binds with low affinity to caldesmon and not to actin under these conditions. Second, the S1 preparation contains a fraction of damaged S1 which binds to actin strongly even in the presence of ATP. In this case, increasing the concentration of S1 would not drive the equilibrium to completion, but would increase the total amount of damaged S1 available for binding. Third, it is experimentally difficult to distinguish between 4 and 0% saturation of actin; it could be difficult to detect displacement of S1, even if it had occurred. The possibilities listed above can be distinguished by examining the stoichiometry of binding of S1 to the caldesmon−actin−tropomyosin complex. A maximum stoichiometry of binding of 1 S1−ATP per 1 actin monomer would be observed if S1 could bind to every actin monomer covered with caldesmon. A 1:1 stoichiometry would also be observed if damaged S1 was responsible for the binding. A maximum stoichiometry of 1:7 would be observed if the S1 were associating with caldesmon and not directly with the actin. A maximum stoichiometry of 1 S1 per 1−7 actin monomers would indicate that one or more actin monomers in the unit of 7 covered by caldesmon could bind to S1−ATP. This possibility could not be eliminated by binding studies of this type. If the lack of effect of caldesmon on binding was due to the inability to discriminate among low levels of bound S1, the effectiveness of caldesmon in displacing S1 should increase as the initial saturation of actin with S1 increases. However, in that case, all of the S1 would be displaced from actin at a sufficiently high caldesmon concentration.

The stoichiometry of S1−ATP binding could be determined by examining the effect of caldesmon on S1−ATP binding at different free S1 concentrations. The most straightforward way of varying the free S1 concentration without causing ATP depletion was to modify the S1 with [^{14}C]ρPDM. This modification produced an S1 species that had about twice the affinity but only a small fraction of the ATPase activity of unmodified S1−ATP. More importantly, the complex of ρPDM−S1 with actin or actin−tropomyosin−troponin is very similar to that formed with S1−ATP (*38*, *41*).

Table 1: Inhibition of Actin-Activated ATPase Rate of ρPDMS1 or Mixtures of ρPDMS1 and S1 by Caldesmon

	ATPase rate (s^{-1})[a,b]	
caldesmon (μM)	ρPDMS1 alone	ρPDMS1 + S1
0	0.013 (1)	0.067 (1)
1	0.008 (0.62)	0.007 (0.1)
2	0.005 (0.38)	0.001 (0.015)

[a] Values in parentheses are normalized to the rate in the absence of caldesmon. [b] Reactions contained 4 μM actin, 0.86 μM smooth tropomyosin, 6 μM ρPDM−S1 in the presence or absence of 0.1 μM S1. The conditions were 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 10 mM potassium propionate, 0.5 mM EGTA, and 2 mM Mg-ATP; 25 °C.

Experiments with ρPDM−S1 would be difficult to interpret if there was significant contamination with unmodified S1; the unmodified S1 would make the greatest contribution to the ATPase activity while the ρPDM−S1 would make the largest contribution to the binding. A significant amount of unmodified S1 would result in an exaggerated difference in the sensitivity of ATPase activity and S1 binding to caldesmon. We therefore removed unmodified S1 following treatment with ρPDM by sedimentation with actin at high ionic strength. The unmodified S1 binds selectively under these conditions. To verify the success of this procedure, we compared the effect of caldesmon on the ATPase activity of ρPDM−S1 alone to that on a mixture of modified and native S1. Since ρPDM−S1 binds with about a 2-fold higher affinity than S1−ATP, we would expect that more caldesmon would be required to inhibit ATPase activity resulting from ρPDM−S1 than from native S1. Table 1 shows that 50% inhibition of ATPase activity occurred at a caldesmon concentration of 1.2 μM for ρPDM−S1. However, 0.4 μM caldesmon was required for 50% inhibition of ATPase activity for a mixture of ρPDM−S1 and S1 indicative of the dominating effect of the more active ATPase activity of unmodified S1. This shows that the ATPase activity that was detected with the pure pPDMS1 was due to ATP hydrolysis catalyzed by pPDMS1 and not due to any contaminating unmodified S1.

Figure 2 shows the effect of caldesmon on the binding of ρPDM−S1 and the ATPase activity at 57 mM ionic strength and 25 °C. In this experiment, the initial fraction of actin containing bound ρPDM−S1 was 0.185 and was reduced to 0.045 at the highest caldesmon concentrations used. This end point corresponds to about 1 bound S1 per 20 actin monomers. The ATPase activity was inhibited to a slightly greater extent than was the binding of ρPDM−S1 to actin−tropomyosin. Comparison of Figures 1 and 2 indicate that the amount of S1 bound to caldesmon−actin−tropomyosin is less than 1 S1 per 5 actin monomers and greater than 1 per 25 actin monomers. The inset to Figure 2 shows an experiment where the initial fraction of actin with bound ρPDM−S1 was 0.17. The addition of caldesmon had a moderate effect on the ATPase activity but only partially displaced the bound ρPDM−S1. There was initially one molecule of S1 bound for each 6 actin monomers, and this was reduced to 1 S1 per 7 actin monomers at high caldesmon

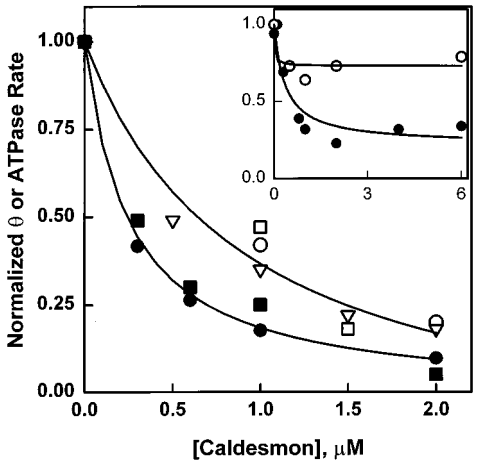

FIGURE 2: Inhibition of binding (open symbols) and actin-activated ATPase activity (closed symbols) of ρPDM−S1 by caldesmon at $\mu = 57$ mM, 25 °C, and high ρPDM−S1 concentration. The assay contained 4 μM actin, 0.86 μM smooth tropomyosin, and 6 μM ρPDM−S1 or [^{14}C]ρPDM−S1 in a buffer composed of 5 mM imidazole, pH 7.0, 2.5 mM $MgCl_2$, 10 mM potassium propionate, and 7.5 mM Mg-ATP. Different symbols represent different experiments with different protein preparations. The value of θ was reduced from 0.185 to 0.04−0.05. The ATPase rate was reduced from 0.025 to 0.0035−0.004 s^{-1}. The inset shows one trial in which the initial value of theta was 0.17 and which was less responsive to caldesmon. The ATPase rate for the inset was reduced from 0.03 to 0.01 s^{-1}. Neither the binding nor the ATPase activity changed with further increases of caldesmon to 8 μM (not shown).

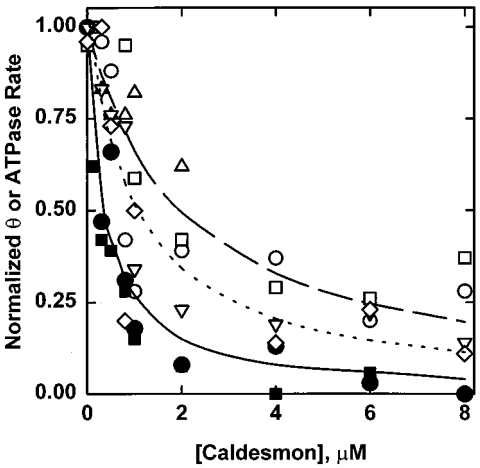

FIGURE 3: Inhibition of binding (open symbols) and actin-activated ATPase activity (closed symbols) of ρPDM−S1 by caldesmon at $\mu = 32.5$ mM, 25 °C. The reactions contained 4 μM actin, 0.86 μM smooth tropomyosin, 6 mM ρPDM−S1, or [^{14}C]ρPDM−S1. Final buffer conditions were 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, 10 mM potassium propionate, 0.5 mM EGTA, and 2 mM Mg-ATP. The initial θ ranged from 0.3 to 0.4 and was reduced to 0.11 to 0.14 at high caldesmon. The maximum ATPase rates were 0.04−0.06 s^{-1}, and the minimum rate was 0−0.003 s^{-1}. Data from individual experiments are shown by different symbols. The effect of caldesmon on ATPase activity (solid line) was the same for all experiments. The effect of caldesmon on binding varied with the protein preparation and followed either the dashed or dotted lines.

concentrations. This effect shown in the inset is similar to that seen with the unmodified S1 in Figure 1.

To further increase the initial fraction of actin containing bound ρPDM−S1, the ionic strength was reduced to 32.5 mM. Figure 3 shows four experiments with different preparations of proteins in which the fraction of actin containing bound ρPDM−S1 was 0.3 (open squares), 0.35

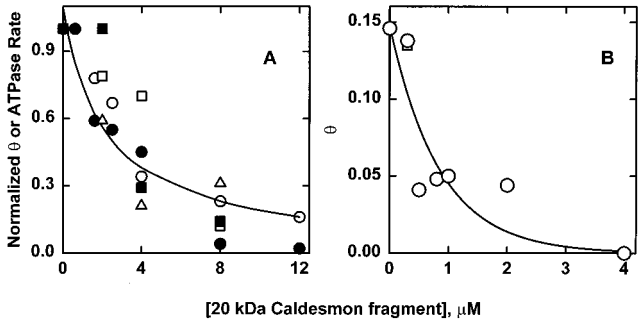

FIGURE 4: The inhibition of binding (open symbols) and actin-activated ATPase activity (closed symbols) of ρPDM−S1 by 20 kDa caldesmon fragment at high ρPDM−S1 concentration. The assay contained 4 μM actin, 0.86 μM smooth tropomyosin, and 6 μM ρPDM−S1 or [^{14}C]ρPDM−S1. (A) $\mu = 32.5$ mM with the same buffer as in Figure 3. The value of θ was reduced from 0.29 to 0.006, while the actin-activated ATPase data was proportionally reduced from 0.06−0.07 to 0.005 s^{-1}. (B) $\mu = 57$ mM with the same buffer as in Figure 2.

(open diamonds), or 0.38 (open circles and triangles) in the four experiments. At this higher initial level of saturation of actin, inhibition of binding of ρPDM−S1 in the presence of caldesmon always occurred. However, the ATPase activity was more sensitive to caldesmon than was the binding of ρPDM−S1 to actin−tropomyosin. As in Figure 2, the amount of ρPDM−S1 bound to actin did not decrease to zero at high caldesmon concentrations. The limiting values of saturation with actin were 0.08 and 0.04 for the binding curves shown. This represents the residual binding of 1 molecule of S1 for each 12 to 25 actin monomers. The highest value measured at high caldesmon (open square) is equivalent to 1 molecule of S1 bound per 10 actin monomers.

The COOH-terminal 20 kDa region of caldesmon binds to actin with a stoichiometry of one 20 kDa fragment per two actin monomers, it inhibits the actin activated ATPase activity of S1, and it lacks the NH_2-terminal caldesmon binding site for myosin (*28*). Figure 4a shows much closer agreement between the inhibition of ATPase activity and inhibition of ρPDM−S1 binding to actin−tropomyosin at 32.5 mM ionic strength than seen with intact caldesmon. Figure 4b demonstrates that the 20 kDa fragment of caldesmon was also an effective inhibitor of ρPDM−S1 binding at 57 mM ionic strength. Under the same conditions, intact caldesmon had little effect on S1 binding in some instances (compare to Figure 2 inset).

DISCUSSION

The results of this study indicate that small amounts of skeletal myosin S1 are able to associate with the caldesmon−actin−tropomyosin complex without the increase in ATPase activity that normally accompanies binding to actin. At high initial levels of binding of S1 to actin−tropomyosin, the addition of caldesmon reduced the value of θ to 0.14 or less. If the initial level of saturation of actin with S1 was less than 0.14, little displacement of S1 was observed in sedimentation assays. The maximum amount of observed residual S1 bound was 1 molecule of S1 per regulatory unit of 7 actin monomers (Figure 2 inset). It appears that S1 is not able to bind to every actin monomer that is covered by caldesmon. Rather, caldesmon displaces most of the bound S1 but a small amount of S1, roughly equal to 1/7 of the

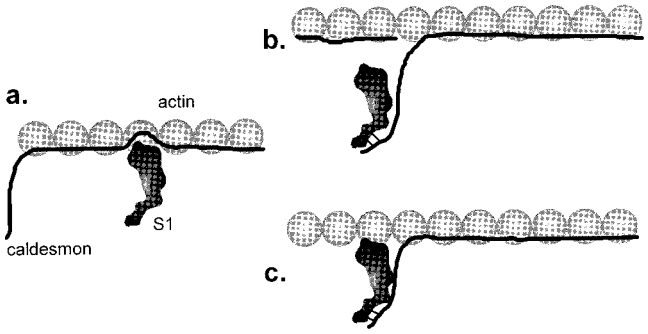

FIGURE 5: Models of complexes between caldesmon, S1, and actin giving 1 bound S1 per caldesmon−actin complex. In (a), caldesmon binds differently to each actin monomer so that 1 actin is free to bind to S1. In (b), S1 cannot bind to any actin that has bound caldesmon. The S1 can bind to the caldesmon, however. (c) is the same as case (b) except that there is not sufficient caldesmon to cover all actin monomers. In this case, the productive attachment of S1 to actin near the end of the caldesmon molecule is increased because of favorable interactions to both actin and caldesmon. There is experimental evidence for (b) and (c).

total concentration of actin monomers, may remain bound to actin−tropomyosin.

The observed stoichiometry of binding of S1 to caldesmon−actin−tropomyosin rules out models in which S1 can bind to every actin monomer that is covered by caldesmon. It is also unlikely that the single S1 bound per 7 actin monomers is due to a fraction of "dead" S1 which forms a rigor bond with actin in the presence of ATP. In the experiments with pPDM−S1, ATP-resistant S1 was removed prior to experimentation as described in Materials and Methods.

The likely remaining explanations for the association of 1 molecule of S1 per 7 actin monomers in an actin−tropomyosin−caldesmon complex are shown in Figure 5. One possibility (Figure 5a) is that observed binding is to a fraction of actin sites (1 of 7) that are able to bind both caldesmon and S1 as in our mosaic multiple binding model (14). If this is true, then the region of caldesmon which is permissive for S1 binding is outside of the COOH 20-kDa region, since Figure 4 showed that the 20 kDa fragment inhibited both S1 binding and ATPase. Note, however, that Redwood and Marston (42) observed inhibition of ATPase without inhibition of binding using a similar 20 kDa caldesmon fragment. Their fragment was recombinant, whereas ours was generated from a chymotryptic digestion. We have been unable to determine the reason for this difference. Another possibility (Figure 5b,c) is that S1 cannot bind to actin monomers that are also bound to caldesmon. The residual binding of S1 in that case results from the low affinity binding of S1 to the NH_2-terminal region of caldesmon (*25*, *26*).

If the mosaic multiple binding model of Figure 5a is correct, then the inhibition of actin-activated ATPase activity would result from a competitive mechanism for 6/7 of the actin monomers and from a noncompetitive mechanism for 1/7 of the actin monomers. In the case of tethering of S1 by caldesmon, shown in Figure 5b,c, the inhibition would be purely competitive. Unfortunately, the reported parameters from steady-state kinetic measurements vary greatly. One laboratory reported that the inhibition was totally noncompetitive (*43*, *29*). Another group reported results which are consistent with 50% of the total inhibition resulting from noncompetitive inhibition (*20*) while we reported earlier that 8% of the total inhibition resulted from noncompetitive inhibition (*44*). Despite the variability in these reports, additional data suggests that noncompetitive inhibition is at best a minor part of the regulation by caldesmon. When caldesmon was placed into a psoas muscle fiber, there was no indication of an effect on the kinetics of cross-bridge cycling (*23*), but troponin was also present in this system and it is possible that small effects on kinetics could have been masked. Furthermore, our preliminary data indicate that caldesmon does not inhibit the rate constant for product release from S1-actin-tropomyosin (A. Resetar and J. M. Chalovich, unpublished). Nevertheless, it is impossible to distinguish between models shown in Figure 5a,b on kinetic grounds.

The available binding data favor a model in which S1 is associated with caldesmon as in Figure 5b,c and not directly with actin. For example, the addition of nucleotide free S1 to actin prevents the fluorescence change that normally occurs when fluorescently labeled caldesmon binds to actin (*45*). Unlike sedimentation assays which cannot discriminate between an caldesmon−actin−S1 complex and one in which the S1 is tethered via caldesmon to actin, the fluorescence change can occur only with direct contact between caldesmon and actin. More recent measurements of the association of both fluorescently labeled S1 and caldesmon to actin under rigor conditions give no indication of direct attachment of S1 to actin in the presence of saturating caldesmon (*46*).

An interesting feature of the binding of S1 to the NH_2-end of caldesmon is that this interaction could actually enhance the productive interaction between S1 and actin under conditions where the caldesmon is subsaturating as shown in Figure 5c. Evidence for such an enhancement in binding has been seen experimentally as an actual increase in the ATPase activity at very low concentrations of caldesmon (*47*) and also as an increase in motility in the in vitro motility assay at low caldesmon concentrations (*24*, *48*). Other examples of an increase in activity in the presence of caldesmon exist (*49*, *51*). Thus, whether caldesmon is inhibitory or stimulatory of actomyosin mediated motility depends on the concentration of caldesmon and on other factors which alter affinity of caldesmon with myosin such as caldesmon phosphorylation (*52*, *53*).

In summary, caldesmon binding to actin−tropomyosin is competitive for the binding of myosin S1−ATP to at least 6 of the 7 actin monomers in an actin−tropomyosin unit. It is most likely that the single S1−ATP bound per actin−tropomyosin−caldesmon unit is attached to caldesmon. If the actin is not saturated with caldesmon, S1−ATP may bind to bare actin monomers and the binding to bare sites adjacent to caldesmon units may be actually favored due to simultaneous interactions among S1−ATP, actin, and caldesmon. The demonstration that approximately 1 S1 may bind per 7 actin monomers in the presence of caldesmon may clarify some of the conflicting reports of the mechanism of caldesmon function. Experiments in progress will serve to characterize the site of S1 binding to actin−caldesmon and examine the impact of this interaction on the observed kinetics of ATP hydrolysis.

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