Characterization of Calponin Binding to Actin†

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ABSTRACT: Calponin, a protein isolated from smooth muscle and nonmuscle cells, has previously been shown to inhibit the actin-activated ATPase activity of myosin. Reports of the stoichiometry of binding range from 1 calponin per actin to 1 calponin per 3 actin monomers. We now report a detailed study of the binding of [^{14}C]iodoacetamide-labeled calponin to actin. The labeling procedure did not significantly alter the binding constant of calponin to actin. The stoichiometry of binding was variable and dependent on ionic strength. Below 110 mM ionic strength, the stoichiometry of binding was 1:1. As the ionic strength was increased above 110 mM ionic strength, the stoichiometry shifted from 1:1 to 1 calponin per 2 actin monomers. At physiological ionic strength, the binding exhibited a small degree of positive cooperativity and was adequately described by a single class of binding sites with an association constant of 6×10^6 M^{-1}. The affinity decreased to 20% of this value in the presence of ATP. Irrespective of the ionic strength, actin formed bundles when saturation with calponin exceeded about 30%. Measurements of the rate of association were complicated by this bundling, but the upper limit for this reaction was placed at 10^6 M^{-1} s^{-1}. The addition of calponin to actin−caldesmon complexes caused displacement of the caldesmon.

Phosphorylation of the 20-kDa myosin light chain by Ca^{2+}−calmodulin-dependent myosin light kinase is thought to activate smooth muscle contraction (for reviews, see Adelstein & Sellers, 1987; Murphy, 1989). Two smooth muscle actin binding proteins, caldesmon and calponin, may also be involved in regulation of contraction (for reviews, see Sobue & Seller, 1991; Marston & Redwood, 1991; Chalovich, 1992). Both proteins inhibit the actin-activated myosin ATPase activity (Chalovich *et al.,* 1987; Winder & Walsh, 1990; Marston, 1991), inhibit relative movement of actin and myosin in *in vitro* motility assays (Shirinsky *et al.,* 1992; Haeberle *et al.,* 1992a; Haeberle, 1994; Horiuchi & Chacko,1995; Kolakowski *et al.*,1995), and when added to skinned muscle fiber preparations alter the contractility of both smooth (Szpacenko *et al.,* 1985; Katsuyama *et al.,* 1992; Pfitzer *et al.,* 1993; Itoh *et al.,* 1994; Obara *et al.,* 1995) and skeletal muscle fibers (Taggart & Marston, 1988; Brenner *et al.,* 1991; Heizmann *et al.,* 1994; Kraft *et al.,* 1995).

Calponin was originally isolated as an actin binding protein (Takahashi *et al.,* 1986). Calponin was later shown to interact with several other proteins including myosin (Lin *et al.,* 1993; Szymanski & Tao, 1993), tropomyosin (Vancompernoll *et al.,* 1990; Childs *et al.,* 1992), Ca^{2+}−calmodulin (Wills *et al.,* 1993; Winder *et al.,* 1993), and other Ca^{2+}-binding proteins (Wills *et al.,* 1994). Interestingly, caldesmon binds to these same proteins. The binding to actin is perhaps the key feature of calponin since this results in attenuation of the ATPase activity of myosin. Therefore, to understand the function of calponin, it is essential to have detailed knowledge of the interaction between actin and calponin.

Several groups have investigated the binding of calponin to actin, but the results are somewhat varied. Winder *et al.* (1991) and Takahashi *et al.* (1986) showed that, in the presence and absence of smooth muscle tropomyosin, calponin binds to skeletal muscle actin with a stoichiometry of 1 calponin molecule per 3 actin monomers at ~120 mM ionic strength. In contrast, Makuch *et al.* (1991) and Kolakowski *et al.* (1995) reported a 1:1 stoichiometry at both 60 and 120 mM ionic strength. The reported association constant ranges from 10^6 M^{-1} to 10^7 M^{-1} (Winder *et al.,* 1991; Nakamura *et al.,* 1993). The determination of the binding parameters for actin binding proteins has been notoriously difficult, especially for those proteins for which a single binding site consists of more than a single actin monomer, as was the case for caldesmon. Such binding is complicated by cooperativity and the "parking problem" (McGhee & von Hippel, 1974) and sometimes by bundling of actin filaments (Pollard & Cooper, 1982). We have now studied the calponin−actin interaction in a more quantitative manner by introducing a [^{14}C] label into the calponin. This allowed a more precise determination of the bound calponin than techniques based on scanning polyacrylamide gels. The data were analyzed by the model of McGhee and von Hippel (1974). The binding was affected by both ionic strength and ATP. The stoichiometry, in particular, was found to vary with ionic strength. The binding of calponin to actin was found to cause aggregation of actin when the saturation of actin was greater than 30%. However, the observed ionic strength dependent change in stoichiometry could not be attributed to this aggregation.

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MATERIALS AND METHODS

Skeletal muscle myosin was prepared from the back and leg muscle of rabbits (Kielley & Harrington, 1960). S-1[1] was prepared from digestion of myosin with chymotrypsin, and the A1 and A2 isozymes were separated by DEAE-Sephacel chromatography (Weeds & Taylor, 1975). Skeletal actin was isolated from rabbit back and leg muscles by the method of Spudich and Watt (1971) as modified by Eisenberg and Kielley (1972). Smooth muscle actin was isolated from fresh chicken gizzards as described by Strzelecka-Golaszewaka *et al.* (1980) and further purified by AcA-54 gel chromatography (Spectrum). Tropomyosin was prepared from turkey gizzards as described by Bretscher (1984). Calmodulin was purified from porcine brains by the method of Yazawa *et al.* (1980). Caldesmon was purified from turkey gizzards by a modification of the method of Bretscher (1984) as described previously (Velaz *et al.*, 1989). Smooth muscle α-actinin was a gift from Dr. Keith Burridge (University of North Carolina, Chapel Hill). Calponin was prepared by a modification of the method described by Abe *et al.* (1990). Briefly, fresh turkey gizzards were cleaned, minced, and homogenized on the middle setting of a Polytron homogenizer in 3 volumes of buffer A (50 mM imidazole-HCl, pH 6.9, 300 mM KCl, 1 mM dithiothreitol, 10 μg/mL leupeptin, 0.5 mM phenylmethanesulfonyl fluoride, 50 μg/mL soybean trypsin inhibitor). The homogenate was heated in a boiling water bath for 8−10 min and cooled on ice for at least 30 min. After centrifugation to remove denatured proteins, the calponin was collected as a 0−30% ammonium sulfate fraction. The ammonium sulfate precipitate was dissolved in buffer B (20 mM sodium acetate, pH 5.6, 6 M urea, 0.1 mM EGTA, 0.01% sodium azide, 0.5 mM dithiothreitol) and dialyzed against the same buffer. The solution was clarified by centrifugation for 30 min at 45 000 rpm in a 50.2 Ti rotor and loaded onto a CM-Sephadex C-50 (1.5 × 16 cm) column equilibrated with buffer B. After washing the column thoroughly, the calponin was eluted with buffer B containing a total of 300 mM NaCl. Fractions containing calponin were dialyzed sequentially against buffer B containing 3 M urea and buffer C (20 mM imidazole hydrochloride, pH 7.2, 400 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.01% sodium azide). The calponin was chromatographed on an AcA-54 column (1.7 × 90 cm) equilibrated with buffer C. Pure calponin was found in the second peak.

Most protein concentrations were determined by absorbance at 280 nm. Caldesmon and calponin concentrations were determined by Lowry assay (1951) with bovine serum albumin as a standard. Because the true calponin concentration is required to accurately determine the stoichiometry of binding, we also measured the calponin concentration by absorbance measurements at 280 nm using the extinction coefficient 0.74 mL/(mg·cm) (Stafford *et al.*, 1995a). The agreement between the two assays was excellent. The extinction coefficient gave a protein concentration 96% of that of the Lowry value. The molecular weights used for calculation of protein concentration were as follows: S-1 (120 000); actin (42 000); tropomyosin (68 000); caldesmon (90 000) (Stafford *et al.*, 1995b); and calponin (32 300) (Takahashi & Nadal-Ginard, 1991; Nishida *et al.*, 1993; Strasser *et al.*, 1993).

Gel Electrophoresis. Protein purity was confirmed by Laemmli type electrophoresis (1970) on the Phast Gel system (Pharmacia) with 8−25% gradient gels.

ATPase Assays. ATPase rates were measured at 25 °C by the liberation of $[^{32}P]P_i$ from [γ-^{32}P]ATP (Chalovich & Eisenberg, 1982). Each assay consisted of a minimum of three time points taken during the initial 20% of the reaction.

Bundling Assays. The bundling of actin filaments by calponin was estimated by a low speed sedimentation assay (Pollard & Cooper, 1982; Chalovich *et al.*, 1987) under the same conditions used for the binding studies. The protein mixtures were incubated for 1 h at either 4 or 25 °C and sedimented by low speed centrifugation. A constant volume of supernatant from each calponin−actin mixture was electrophoresed on an 8−20% polyacrylamide gradient gel in the presence of SDS. The Coomassie blue stained gel was scanned with an HP ScanJet IIcx scanner having an HP C2501A transparency adapter (Hewlett-Packard). The concentration of actin in each lane was quantified with the Image Quant program (Molecular Dynamics).

Binding Assays. Calponin was reacted with a 2-fold molar excess of [^{14}C]iodoacetamide in a buffer containing 84 mM NaCl, 7.2 mM $MgCl_2$, 15.6 mM imidazole, pH 8.0, and 0.5 mM EGTA for 4 h at 18 °C. The reaction was terminated by the addition of dithiothreitol to a final concentration of 1 mM. Unreacted [^{14}C]iodoacetamide was removed by extensive dialysis. Typically, 0.8 ± 0.2 mol of label was incorporated per mole of calponin. The binding of [^{14}C]-labeled calponin was determined by the centrifugation method described by Velaz *et al.* (1989). Prior to each experiment, the [^{14}C]-labeled calponin was clarified by centrifugation for 30 min at 45 000 rpm in a 50Ti rotor. Binding data were corrected for the following: (1) the fraction of calponin which did not bind at very high actin concentrations (18−22%), (2) nonspecific sedimentation of calponin in the absence of actin (4−10%), and (3) the fraction of the actin which did not sediment (5%). All binding constants are expressed as association constants.

Fluorescent Probes. Calponin and caldesmon were labeled with a 7.2-fold molar excess of IANBD (25 mM stock in dimethyl formamide) in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA for 4 h at 18 °C. Reactions were stopped with 1 mM dithiothreitol, and unreacted IANBD was removed by gel filtration on an AcA-202 column. Before use, the protein was clarified by centrifugation for 30 min at 45 000 rpm in a 50Ti rotor. The concentration of the label was determined from a molar absorbance at 495 nm of 2.6×10^4 M^{-1} cm^{-1} (Trayer & Trayer, 1988). The extent of labeling was as follows: 1.7 ± 0.2 probe per mole of caldesmon and 1.3 ± 0.1 probe per mole of calponin. Skeletal muscle actin was labeled with acrylodan at Cys 374 by incubating at 4 °C for 12 h in a solution containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 1 mM sodium azide and 5-fold molar excess of acrylodan. The extent of labeling was 70−100% and was determined from a molar absorbance at 360 nm of 1.29×10^4 M^{-1} cm^{-1} (Prendergast *et al.*, 1983).

[1] Abbreviations: S-1, myosin subfragment 1; HMM, heavy meromyosin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; IANBD, 4-[*N*-((2-(iodoacetoxy)ethyl)-*N*-methylamino]-7-nitrobenz-2-oxa-1,3-diazole; caldesmon-NBD, IANBD-labeled caldesmon; calponin-NBD, IANBD-labeled calponin; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; actin-acrylodan, acrylodan-labeled actin; MIANS, 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid.

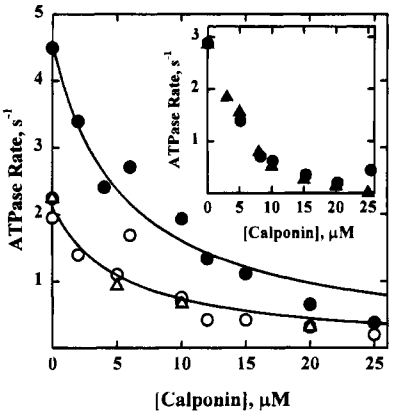

FIGURE 1: Effect of calponin and [^{14}C]-labeled calponin on acto-S-1 ATPase activity. Measurements were made at 25 °C in solutions containing 42 mM NaCl, 4.8 mM $MgCl_2$, 10 mM imidazole, pH 7.0, 0.25 mM EGTA, 1 mM dithiothreitol, 0.3 μM skeletal muscle (A1)S-1, 20 μM skeletal muscle actin, and varied concentrations of unmodified (circles) and [^{14}C]-labeled (triangles) calponin in both the absence (open symbols) and presence (closed symbols) of 4.3 μM smooth muscle tropomyosin. Rates were corrected for hydrolysis in the absence of actin (0.084 s^{-1}). The inset contains data from a different experiment which shows that tropomyosin exhibits its normal effect on the ATPase rate of the modified calponin.

Stopped Flow Experiments. Measurements were made at 15 °C with an Applied Photophysics DX17.MV/2 sequential stopped flow spectrofluorometer. IANBD fluorescence was monitored through a filter with 0% transmission at 510 nm and 80% transmission at 540 nm with excitation at 492 nm. Acrylodan fluorescence was monitored through a filter with 0% transmission at 430 nm and 80% transmission at 500 nm with excitation at 391 nm. The curves were fitted to the nonlinear Levenberg–Marquardt algorithm (software from Applied Photophysics).

Mathematical Modeling. Simulations were made with the mathematics program Mathematica (Wolfram Research). Mathematical models were fitted to the data with the MLAB mathematical modeling program (Civilized Software, Inc.). Because the actin binding site for calponin may consist of more than a single actin monomer, the Scatchard equation is not an appropriate model for analyzing the binding data. The model of McGhee and von Hippel (1974) is suitable for the binding of a ligand to a long lattice where a single ligand binding site consists of n actin monomers, where $n \geq 1$. When $n > 1$, binding of ligands requires that a space consisting of at least n consecutive actin monomers is available. At high levels of saturation, additional binding of ligand is contingent on rearrangement of bound ligands to create blocks of n actin monomers. The equation is:

$$\nu/L = K(1 - n\nu)\{[(2\omega - 1)(1 - n\nu) + \nu - R]/[2(\omega - 1)(1 - n\nu)]\}^{n-1}\{[1\ (n + 1)\nu + R]/[2(1 - n\nu)]\}^2$$

where

$$R = \{[1 - (n + 1)\nu]^2 + 4\omega\nu(1 - n\nu)\}^{1/2}$$

In this equation, ν is the ratio of ligand bound to total actin, L is the free ligand concentration, K is the affinity of a single ligand to an isolated binding site, n is the number of subunits or actin monomers making a single ligand binding site, and ω is the cooperativity parameter between adjacent ligands.

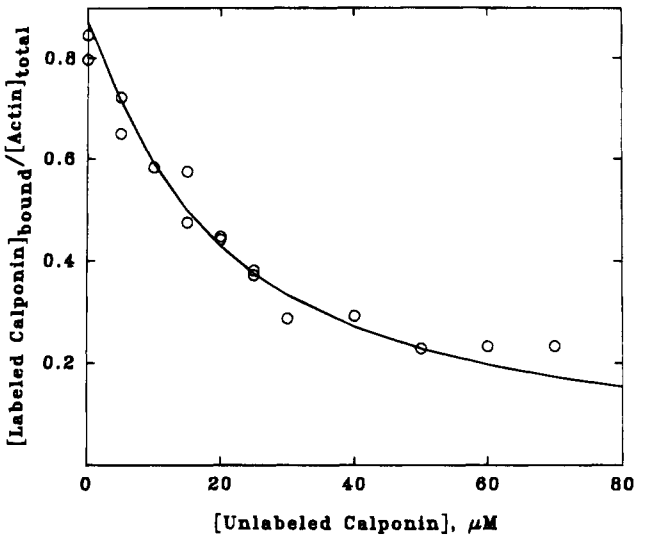

FIGURE 2: Competitive binding of unmodified and modified calponin to actin. Binding was measured in the presence of 15 μM skeletal muscle actin, 20 μM [^{14}C]-labeled calponin, and varied concentrations of unmodified calponin as described in Figure 1. The theoretical curve was drawn assuming a single class of binding sites with 1:1 stoichiometry of calponin binding to actin. The fitted association constants of unmodified and [^{14}C]-labeled calponin binding to actin are 1.43×10^6 M^{-1} and 1×10^6 M^{-1}, respectively.

RESULTS

The effects of calponin on the acto-S-1 ATPase activity and the effect of modification with iodoacetamide were first determined. Figure 1 shows that unmodified calponin inhibits the ATPase activity by approximately 85% of the initial value in both the presence and absence of tropomyosin. The percent inhibition at each calponin concentration is the same in the presence and absence of tropomyosin. Therefore, tropomyosin has no effect on the inhibitory function of calponin although it enhances the ATPase rate. Modification of calponin with [^{14}C]iodoacetamide had no effect on the ability of the calponin to inhibit ATPase activity. Figure 1 shows that the concentration dependence of the ATPase activity is identical for modified and unmodified calponin in the absence of tropomyosin. The inset to Figure 1 shows the effect of modified and unmodified calponin on ATPase activity in the presence of tropomyosin; the results are identical.

As a further test for changes in affinity to actin, a binding competition experiment between unmodified and [^{14}C]-labeled calponin was done. Figure 2 shows the displacement of [^{14}C]-labeled calponin from actin by unlabeled calponin under conditions where the actin was nearly saturated with [^{14}C]-labeled calponin. The theoretical curve was calculated by assuming a single class of binding sites with association constants for unmodified and [^{14}C]-labeled calponin binding to actin equal to 1.43×10^6 M^{-1} and 1×10^6 M^{-1}, respectively. Judging from the competition and ATPase assays, the [^{14}C]-labeled calponin is a good model for studying the binding of calponin to actin.

The binding of [^{14}C]-labeled calponin to actin was measured at several different ionic strengths. Figure 3 shows the binding at the lowest ionic strength studied (61.4 mM). No difference was observed between the binding to skeletal and smooth muscle actin. Furthermore, as observed earlier by Winder *et al.* (1991), the addition of smooth muscle tropomyosin was without effect. At this low ionic strength,

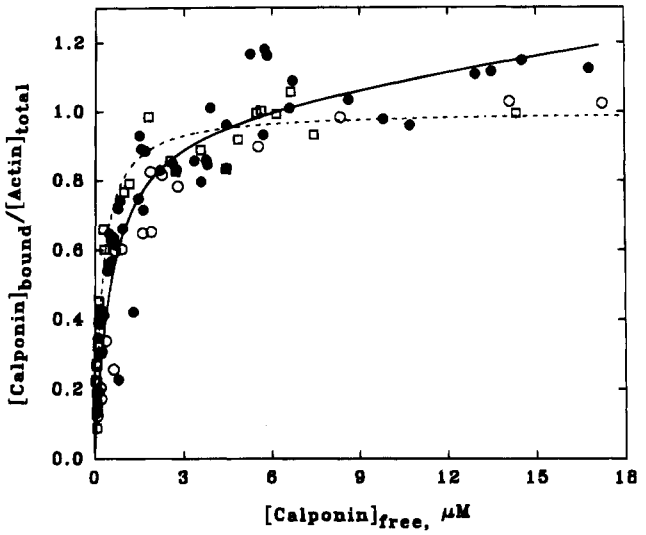

FIGURE 3: Binding of calponin to actin at 61.4 mM ionic strength. Binding of [^{14}C]-labeled calponin was measured at 25 °C in a solution containing 7 or 15 μM skeletal (circles) or smooth muscle actin (squares) in either the absence (open symbols) or presence (closed symbols) of smooth muscle tropomyosin (3.5 mol of actin/mol of tropomyosin). The conditions were the same as in Figure 1. The dashed line is the best fit to a single binding model with $K = 4.4 \times 10^6$ M^{-1}. The solid line is a better fit, which includes a nonspecific binding site. The affinity for the specific site is 1.9×10^6 M^{-1}.

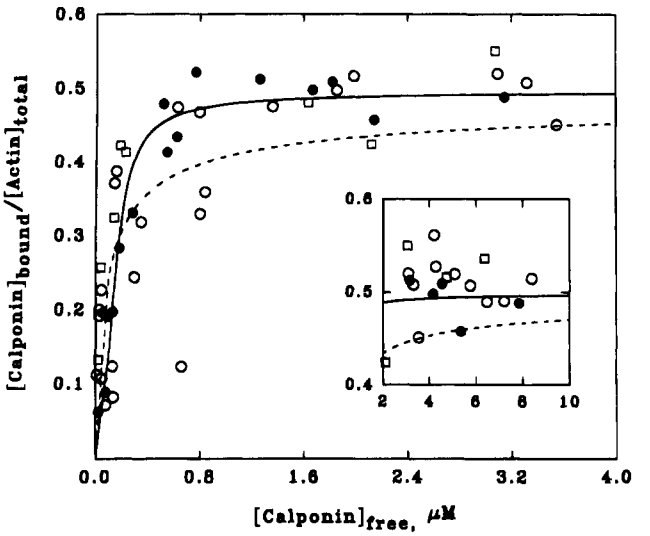

FIGURE 4: Binding of calponin to actin at 150 mM ionic strength. The conditions were the same as in Figure 1 except that potassium propionate was added to 88.6 mM. The solid line is the best fit of the McGhee–von Hippel equation with cooperativity; $K = 5.4 \times 10^5$ M^{-1} and $\omega = 11$. The dashed line is the best fit without cooperativity; $K = 7 \times 10^6$ M^{-1} and $\omega = 1$. The inset shows that the binding does not approach 1:1 stoichiometry even at very high calponin concentrations.

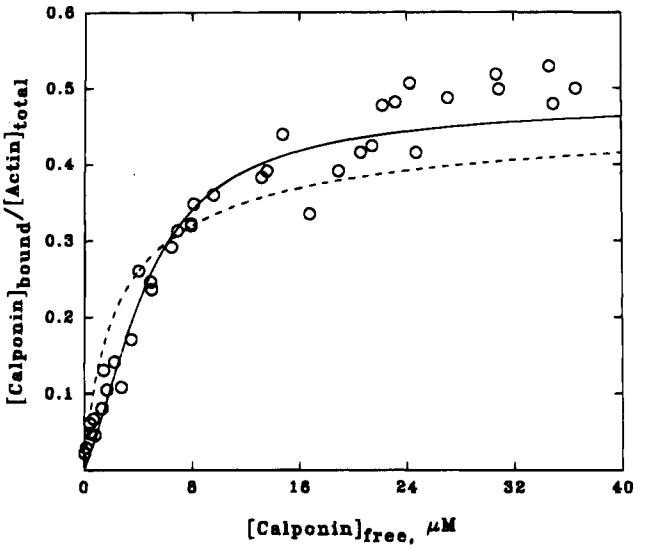

FIGURE 5: Binding of calponin to actin at 250 mM ionic strength. The conditions were the same as in Figure 1 except potassium propionate was added to 188.6 mM. The solid line is the best fit of the McGhee–von Hippel equation with cooperativity; $K = 4.5 \times 10^4$ M^{-1} and $\omega = 4.3$. The dashed line is the best fit without cooperativity; $K = 2.1 \times 10^5$ M^{-1} and $\omega = 1$.

the stoichiometry of binding of calponin to actin was 1:1. Because of the 1:1 stoichiometry, the data were analyzed by either a simple single binding site model (dashed line) or one which had additional nonspecific binding sites on actin (solid line). The model which included nonspecific binding sites had an improved fit. More complex models having two calponin molecules (such as dimers) bound to two actin monomers at two sites with or without additional nonspecific binding sites on actin did not improve the fit.

The binding at 150 mM ionic strength is shown in Figure 4. As before, similar results were obtained with smooth and skeletal muscle actin, and tropomyosin was without effect. However, in contrast to the binding at lower ionic strength, the stoichiometry of binding was 1 calponin per 2 actin monomers. The inset of Figure 4 shows that even at very high calponin concentrations there was no indication of an approach to 1:1 binding. Because two actin monomers formed a single calponin binding site, the analysis of McGhee and von Hippel (1974) was used. The solid line shows the best fit of the model to the data, while the dashed line is the best fit to the data if no cooperativity is allowed, that is, if ω is set to 1. The model incorporating cooperativity more precisely defines the data. Although Figure 4 shows a theoretical curve with ω of 11, a reasonable fit was obtained for any value of ω between 9 and 20.

At the highest ionic strength studied (250 mM), the stoichiometry of binding was again 1 calponin per 2 actin monomers. The results are shown in Figure 5. The solid theoretical curve with $\omega = 4.3$ is a better representation of the data than the curve with no cooperativity (dashed line). The strength of binding of calponin to actin decreased to 3.3% of the initial value when the ionic strength was increased from 150 to 250 mM.

Additional binding experiments were done between 60 and 150 mM ionic strength (data not shown). At 61.4 mM ionic strength, the measured stoichiometry for calponin to actin ranged from 0.99:1 to 1.2:1. The stoichiometry remained 1:1 at 85 mM ionic strength but had changed to 1 calponin per 2 actin monomers at 110 mM ionic strength. The stoichiometry remained 1:2 at 120, 150, and 250 mM ionic strength. Several possible explanations for this change in stoichiometry were evaluated.

Calponin can bundle actin filaments, and the possibility was considered that changes in the extent of bundling were responsible for the apparent change in stoichiometry. We simultaneously measured the binding of [^{14}C]-labeled calponin and the bundling as the concentration of [^{14}C]-labeled calponin was varied. Figure 6 shows that at both 61.4 and 150 mM ionic strength the amount of actin in the supernatant

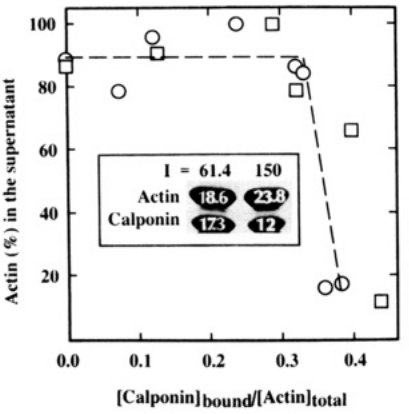

FIGURE 6: Relationship between actin bundling and the amount of calponin bound to actin. The bundling assay was run using 7 μM actin and varied concentrations of [^{14}C]-labeled calponin at 61.4 (squares) or 150 mM ionic strength (circles) as described in Figures 3 and 4, respectively. The amount of calponin bound to actin was simultaneously measured by the high speed sedimentation assay as described in the text. The inset shows the amount of calponin bound to the bundled actin that sedimented at low speed in the presence of a saturating calponin concentration. The concentrations of actin and calponin were 15 and 40 μM, respectively. Lane 1: 61.4 mM ionic strength, the ratio of bound calponin to actin is 1:1.08. Lane 2: 150 mM ionic strength, the ratio of bound calponin to actin is 1:1.98.

decreased when more than 30% of the actin monomers had bound calponin. When θ (calponin bound/actin total) was smaller than ~0.3, the amount of actin in the supernatant remained nearly constant (less than 1.5% sedimentation). In both conditions, the bundling transition occurred over a narrow range of bound calponin. Since the bundling occurred at a given fraction of bound calponin regardless of the ionic strength, it is unlikely that changes in actin bundling resulted in the apparent change in stoichiometry.

We also examined the pellets of the bundled actin to determine the stoichiometry of bound calponin. The inset to Figure 6 shows that, at saturating calponin concentrations, the stoichiometry of bound calponin was again dependent on the ionic strength. At 61.4 mM ionic strength, the stoichiometry was 1 calponin per actin monomer, whereas at 150 mM ionic strength, the stoichiometry was 1:2 just as in the high speed binding assay.

Because of difficulties in preventing bundling, we decided to induce bundle formation with α-actinin (Burridge & Feramisco, 1981; Mayer & Aebi, 1990) and measure the binding of calponin to this organized system. Figure 7 shows the binding of calponin to actin with 1 mol of α-actinin per 205 mole of actin. The affinity of calponin for actin−α-actinin complex (solid line) is not much different than that of actin alone (dashed line). Furthermore, the stoichiometry of binding of calponin to actin was unchanged by the intentional bundling. Because the type of actin bundle formed depends on the ratio of α-actinin to actin (Mayer & Aebi, 1990), the effect of other ratios of α-actinin to actin were investigated. Using very high concentrations of calponin to ensure saturation, the effect of addition of one α-actinin molecule per 30 or 60 actin molecules is also shown in Figure 7. As before, the stoichiometry remained 1 calponin per 2 actin monomers.

We wished to determine if other agents such as ATP might alter the stoichiometry of binding of calponin to actin. Nishida *et al.* (1990) and Makuch *et al.*(1991) reported that

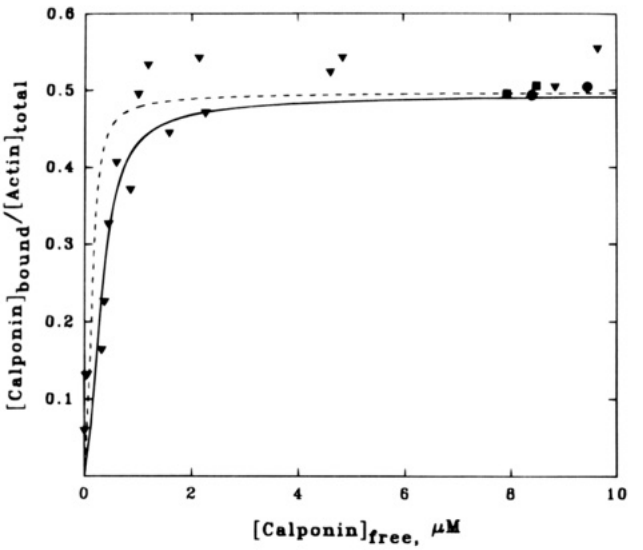

FIGURE 7: Binding of [^{14}C]-labeled calponin to crosslinked actin. Binding was measured in a solution containing 7 or 15 μM skeletal muscle actin−α-actinin complex and varied concentrations of calponin under the conditions of Figure 4. Ratios of smooth muscle α-actinin to actin were 1:205 (solid triangles), 1:60 (solid circles), and 1:30 (solid squares). The best fitted curve (solid line) was generated by the McGhee−von Hippel equation assuming a single class of binding sites with stoichiometry of 1 calponin per 2 actin, an association constant of 3.5×10^5 M^{-1}, and ω of 7.9. The dashed line is taken from Figure 4 with an association constant of 5.4×10^5 M^{-1} and ω of 11.

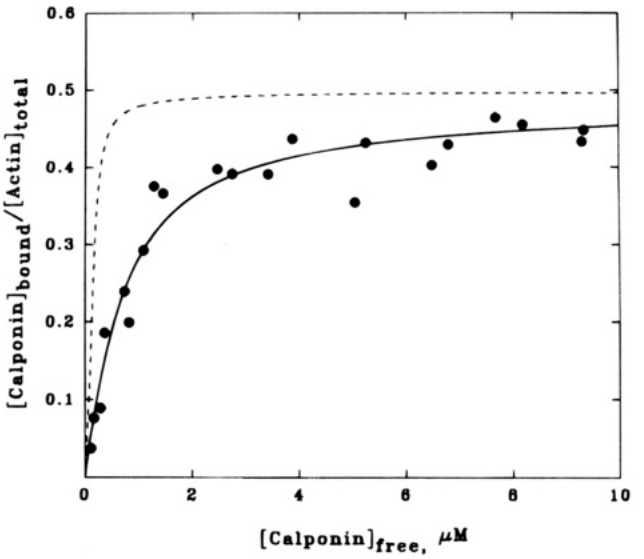

FIGURE 8: Effect of ATP on the binding of [^{14}C]-labeled calponin to actin. Binding was measured in a mixture containing 7 or 15 μM skeletal muscle actin and 2 mM ATP. The best fitted curve (solid line) was determined with the McGhee−von Hippel equation assuming a single class of binding sites with stoichiometry of 1:2, $K = 4.8 \times 10^5$ M^{-1}, and $\omega = 2.3$. The dashed line is taken from Figure 4 with $K = 5.4 \times 10^5$ M^{-1} and ω of 11. The conditions were the same as in Figure 4 except that 2 mM ATP was included.

ATP weakens the binding of calponin to actin. In Figure 8, the binding of calponin to actin in the presence of 2 mM ATP at 150 mM ionic strength is compared to the binding in the absence of ATP. The addition of ATP decreased the association constant to 19% of its initial value, but the stoichiometry of binding of calponin to actin did not change.

An attempt was made to determine the rate of formation of the calponin−actin complex by monitoring the change in fluorescence that occurs when calponin-NBD binds to actin

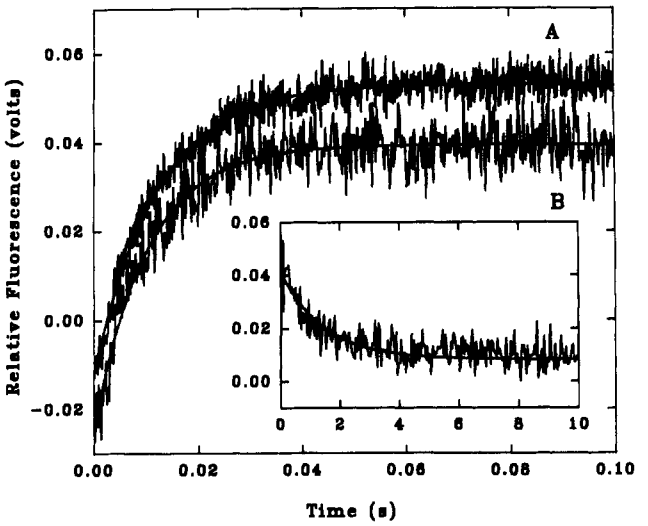

FIGURE 9: Kinetics of binding of caldesmon-NBD to actin in the absence and presence of calponin. In this experiment, 0.8 μM caldesmon-NBD or 0.8 μM caldesmon-NBD and 12 μM calponin were rapidly mixed with an equal volume of 4 μM skeletal muscle actin at 15 °C. The conditions were the same as in Figure 4. The data (average of 8−10 traces) were well fit to a single exponential process. Curve A: caldesmon-NBD mixed with actin; curve B: caldesmon-NBD and calponin mixed with actin in the first 0.1 s. Inset: Long time interval view of curve B showing the subsequent slow replacement of bound caldesmon-NBD with calponin.

or to actin-acrylodan in a stopped-flow spectrophotometer. We first tested the effect of IANBD modification of calponin by using this probe to measure the rate of binding of calponin to Ca^{2+}−calmodulin. A rapid fluorescence change occurred upon binding of calponin-NBD to Ca^{2+}-calmodulin which was satisfactorily fitted by a single exponential function (data not shown). The observed rate constant (4 s^{-1}) agreed quite well with the rate constant (4.4 s^{-1}) reported early for unmodified calponin binding to MIANS-labeled calmodulin (Winder *et al.*, 1993). Both values represent the observed rate at saturating ligand concentrations.

When this same probe was used to study the rate of binding to either actin or actin-acrylodan, the observed transients were very complex. When calponin-NBD was added to actin-acrylodan, there was a rapid decrease in actin fluorescence, but the signal had a small amplitude. This initial phase was not detected when calponin-NBD and actin were mixed together. Several slow phases following the mixing were detected with both actin and actin-acrylodan. In the case of calponin-NBD and actin-acrylodan, the slow phases were accompanied by changes in light scattering. In neither case was there clear evidence that the fluorescence signal reported the binding event. We therefore used an indirect method to obtain an upper limit for the rate of association which was based on an earlier observation that caldesmon and calponin compete for binding to actin (Mukuch et al., 1991).

We had shown earlier that caldesmon-NBD binds to actin-acrylodan with a rate constant of 10^7 M^{-1} s^{-1} (Chalovich *et al.*, 1995). This binding could be measured easily and was not subject to the complexities observed with calponin. In Figure 9, curve A shows the stopped-flow trace for the binding of 0.8 μM caldesmon-NBD to 4 μM skeletal muscle actin (k_{obs} = 83 s^{-1}). When the experiment was repeated in the presence of a large excess (12 μM) of unlabeled calponin, the binding of caldesmon was initially unchanged (curve B; k_{obs} = 87 s^{-1}). This indicated that either the caldesmon binding was not competitive with calponin binding or that caldesmon binding was much faster than calponin binding. Continued observation of the fluorescence over a longer time interval indicated that the second possibility was correct. The inset of Figure 9 shows that the fluorescence decreased slowly as the caldesmon dissociated and was replaced by calponin. Although calponin binds slowly, its binding was thermodynamically favored since the association constants are similar and the calponin was in large excess over the caldesmon. Thus, while caldesmon bound more quickly to the actin, the final state was one in which calponin and not caldesmon was bound to actin.

DISCUSSION

Because of the varied results obtained for the stoichiometry of binding of calponin to actin, we reinvestigated this problem using a sensitive cosedimentation technique with [^{14}C]-labeled calponin. We looked for variables which might influence affinity and stoichiometry of binding. ATP weakened the calponin−actin interaction but did not affect the stoichiometry of binding. Bundling of actin by calponin or α-actinin had no effect on binding. The source of actin had no effect on the binding; the stoichiometry and binding constant remained the same for smooth muscle and skeletal muscle actin. This differs from an earlier report that calponin binds about 7-fold more tightly to smooth muscle actin (Winder *et al.*, 1991). Smooth muscle tropomyosin had no effect on the affinity or stoichiometry of binding nor on the inhibitory activity of calponin. This agrees with other reports that tropomyosin does not affect the inhibitory activity of calponin (Winder & Walsh, 1990; Marston, 1991), the binding of calponin to actin (Winder *et al.*, 1991) nor the inhibition of actin filaments in the *in vitro* motility assay (Shirinsky *et al.*, 1992). The only factor which we found to influence the stoichiometry of binding is the ionic strength. Above 110 mM ionic strength, the stoichiometry of binding was 1 calponin per 2 actin monomers; this probably represents the physiological state.

At ionic strengths ≥110 mM, the binding of calponin to actin was analyzed using the model of McGhee and von Hippel (1974). It is interesting that the value of the positive cooperativity, ω, was small, ranging from 2 to 20. This positive cooperativity is similar to that which we have seen with caldesmon (Velaz *et al.*, 1989) but is much smaller than that which occurs with skeletal muscle tropomyosin (ω = 1600) (Wegner, 1979). Thus, unlike tropomyosin, the interactions among adjacent ligand molecules have little role in the strength of binding.

It is difficult to say with certainty why the stoichiometry of binding of calponin to actin is 1:1 at low ionic strength. Several possible models for the binding of calponin to actin which give 1:1 stoichiometry were examined. These models are illustrated in Figure 10. Model I depicts the most common example of 1:2 binding. Model II is the most common example of 1:1 binding with 1 calponin bound per actin monomer. This could occur if, at low ionic strength, the calponin folded so that one of the sites became unavailable for interaction with actin. In model III, the two calponin molecules bind to two actin monomers at two sites. This would occur if a low affinity, possibly nonspecific, site on actin became occupied at low ionic strength. Finally, in

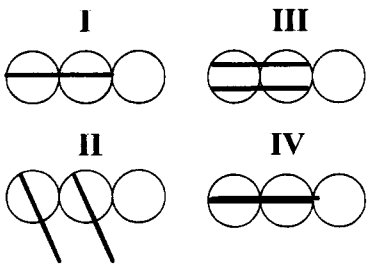

FIGURE 10: Hypothetical models for the binding of calponin to actin. The structure of actin−calponin complex is shown with actin monomers represented as closed circles and calponin represented as black rods. Model I represents the actin−calponin complex formed at ionic strengths >110 mM. Models II−IV represent the actin−calponin complex formed in ionic strength <110 mM.

model IV, the calponin is shown to dimerize at low ionic strength. Models II−IV were fitted to the data in Figure 3 but could not be distinguished.

Kolakowski *et al.* (1995) reported that two types of calponin−actin complexes could form irrespective of the ionic strength. The complex of 1 calponin per 2 actin monomers was found to be insoluble due to bundling of actin filaments. The 1:1 complex, however, was found to be soluble. In contrast, we observed bundling of actin when more than 30% of the actin was bound to calponin regardless of the ionic strength. Yet, the stoichiometry of binding was dependent on ionic strength regardless of the degree of crosslinking. We also repeated our binding studies using low speed sedimentation where only bundled actin was sedimented (Figure 6). We obtained the same stoichiometry of binding for the bundled actin which sedimented at low speed as for the total actin which sedimented at high speed. In both cases, the stoichiometry of binding was dependent on the ionic strength.

A discrepancy which exists with calponin as well as with caldesmon binding to actin is that the stoichiometries obtained with the pure proteins are considerably different from the natural composition of these proteins. Although reported ratios of calponin to actin in smooth muscle thin filaments range from 1:10 (Nishida *et al.*, 1990) to 1:16 (Marston, 1991), all values measured in solution are less than or equal to 1:3. There appears to be a similar discrepancy with caldesmon although this depends somewhat on the values chosen for comparison of the solution (Velaz *et al.*, 1989; Smith *et al.*, 1987) and fiber (Marston, 1990; Haeberle *et al.*, 1992b) results. One possible explanation for this behavior is that other actin binding proteins in the muscle might act as positive or negative effectors for the binding of caldesmon and calponin. For example, caldesmon and calponin compete with each other for binding to actin (Makuch *et al.*, 1991). This has been confirmed by our stopped-flow results of Figure 9. Other actin binding proteins may also be competitive for calponin binding.

Our data also indicate that the rate of binding of calponin to actin is quite slow. While we were unable to measure the binding directly, we were able to determine that calponin binds with a rate that is much slower than that of caldesmon (Figure 9). An exact analysis of the data is complex because each caldesmon binds to 7 actin monomers and each calponin binds to 2 actin monomers and each binding is associated with a "parking problem" and cooperativity. However, it is possible to obtain an estimate of the association constant by a very simple model which treats the caldesmon and calponin as univalent ligands. With this large simplification, the data can be reasonably well described by assuming that calponin binds with an association rate constant near 1×10^5 M^{-1} s^{-1} and a dissociation rate constant of about 0.017 s^{-1}. We hope to be able to refine this estimate in the future. This value is important since it might limit the rate at which calponin could exert its physiological effects if this involves attachment to actin.

In summary, under physiological conditions, a single calponin molecule binds to two actin monomers. This interaction is mainly driven by the binding of calponin to isolated actin units, not by the cooperative interaction of calponin molecules. The affinity is weakened by the addition of ATP, but the stoichiometry is not affected. Neither tropomyosin nor the actin bundling protein α-actinin alters the binding of calponin to actin. Finally, the rate of association of calponin with actin is slower than caldesmon, and caldesmon and calponin compete with each other for actin binding.

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