Calcium-Dependent Regulation of the Caldesmon–Heavy Meromyosin Interaction by Caltropin

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ABSTRACT: The binding of chicken gizzard caldesmon to smooth muscle heavy meromyosin (HMM) was studied using caldesmon–Sepharose 4B affinity chromatography, far-ultraviolet circular dichroism (CD), and the fluorescent probe acrylodan. When HMM was applied to a caldesmon–Sepharose column in the presence of 40 mM NaCl, most of the protein was retained on the column, and HMM could be eluted by increasing the NaCl level to 0.5 M; this interaction was not Ca^{2+}-dependent. Far-UV CD studies indicated an interaction between caldesmon and HMM since the experimentally observed ellipticity values at 222 and 207 nm deviated from the theoretical values for the complex, and this interaction was also not Ca^{2+}-sensitive. Addition of HMM to a caldesmon–caltropin complex induced a conformational change suggesting the formation of a ternary complex for which Ca^{2+} was essential. Acrylodan-labeled caldesmon, when excited at 375 nm, had an emission maximum at 515 ± 2 nm. Addition of HMM resulted in a nearly 20% decrease in fluorescence intensity with little or no shift in the emission maximum. Titration of HMM with labeled caldesmon indicated a strong affinity for HMM [K_a was on the order of $(4.5 \pm 0.5) \times 10^7$ M^{-1}], and this interaction was observed both in the presence and in the absence of calcium. When HMM was titrated with labeled caldesmon in the presence of caltropin in a 0.2 mM Ca^{2+} medium, its affinity for caldesmon was lowered nearly 3-fold [$K_a \approx (1.50 \pm 0.5) \times 10^7$ M^{-1}]. Caltropin, which is very potent in reversing the inhibitory effect of caldesmon in the presence of calcium (Mani et al., 1992), is shown in this study to modulate the interaction between caldesmon and smooth muscle heavy meromyosin, thus making it a potential calcium factor in regulating caldesmon in smooth muscle.

Caldesmon is a major actin binding protein associated with thin filaments of smooth muscle (Sobue et al., 1981; Marston & Lehman, 1985) and nonmuscle cells (Owada et al., 1984). In smooth muscle it is found bound to the thin filaments in the contractile domain where the thick and thin filaments overlap (Furst et al., 1986; Lehman et al., 1987), and its concentration in smooth muscle is believed to be 10 μM (Walsh & Sutherland, 1989). Hence, there is enough caldesmon in the correct position within the contractile apparatus of the intact cell for it to play a major role in smooth muscle contraction. Current studies suggest a vital role for this protein in the regulation of smooth muscle contraction since it inhibits superprecipitation of actomyosin (Sobue et al., 1981) and the actin-activated ATPase[1] activity of myosin (Dabrowska et al., 1985; Smith & Marston, 1985; Moody et al., 1985; Sobue et al., 1985) and its subfragments (Lash et al., 1986; Cholovich et al., 1987). However, defining the precise role of caldesmon in cells requires an understanding of its interactions with several key proteins, namely, actin, myosin, calmodulin, and caltropin.

The ability of caldesmon to bind tightly to both actin and smooth muscle myosin at the same time sets this protein apart from other actin binding proteins. Since actin and myosin bind at different sites on caldesmon, it is able to cross-link these two proteins. Myosin binding ought to be physiologically significant since it can be observed not only with pure caldesmon but also in native thin filaments containing caldesmon. Caldesmon binds to actin with an affinity near 10^7 M^{-1} (Velaz et al., 1989) and to myosin with an affinity near 10^6 M^{-1} (Hemric & Chalovich, 1990). The myosin binding activity has been localized to an N-terminal "27 K" chymotryptic fragment of caldesmon (Velaz et al., 1990), while the actin binding region(s) are largely confined to a C-terminal "35 K" fragment (Szpacenko & Dabrowska, 1986; Fujii et al., 1987). In order to explain the mechanism by which caldesmon inhibits actin activation of myosin ATPase, Hemric and Chalovich (1990) have proposed a model in which caldesmon inhibits the binding of all myosin ATP complexes to actin, thereby inhibiting ATP hydrolysis. Support for this model comes from the fact that both myosin and caldesmon share some of the same binding regions on actin (N-terminal region) as seen by NMR (Levine et al., 1990), cross-linking studies (Bartegi et al., 1990), or competition with antibodies directed against specific regions in actin (Adams et al., 1990). The observation that the C-terminal region of caldesmon, which does not bind to myosin, is able to inhibit ATP hydrolysis gives further credence to the hypothesis that caldesmon is a competitive inhibitor of the binding of myosin to actin (Chalovich et al., 1987). Even though the myosin binding site is thought to be on the N-terminal end of caldesmon, the stoichiometry and the affinity with which it binds to caldesmon are not well established. Quantitation of the binding is important in determining if this association is specific and whether this interaction could occur under *in vivo* conditions on the basic of the affinity with which it binds to caldesmon.

In the present study, the interaction between smooth muscle HMM and caldesmon was investigated using a caldesmon affinity column and spectroscopic techniques including circular dichroism and the fluorescent probe acrylodan. Using this thiol-specific fluorescent probe, we were able to establish the

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; CD, circular dichroism; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; ATPase, adenosine triphosphatase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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stoichiometry and the affinity with which HMM bound to caldesmon. In addition, we have shown that caltropin in the presence of calcium was effective in weakening the interaction between caldesmon and myosin.

MATERIALS AND METHODS

Protein Purification. Caltropin was isolated from chicken gizzard using the procedure described in our earlier paper (Mani & Kay, 1990). F-actin was purified from rabbit skeletal muscle according to Pardee and Spudich (1982). Smooth muscle heavy meromyosin from chicken gizzard was isolated using the procedure described by Suzuki et al. (1985). Caldesmon from fresh or frozen chicken gizzards was prepared by a modification of the method of Bretscher (1984). Initial purification of caldesmon was carried out using an ion-exchange column as described by Clark et al. (1986). The caldesmon fraction obtained from the DEAE-Sephacel column was next applied to a 2.5 × 30 cm column of hydroxylapatite (Bio-Rad) in the presence of 10 mM sodium phosphate, pH 6.5, 0.05% sodium azide, 1 mM DTT, and 0.3 mM $CaCl_2$. A linear gradient was formed from 500 mL of each of 10 and 250 mM sodium phosphate buffers. Caldesmon eluted as a sharp peak around 0.21 M phosphate and was homogeneous when tested by 12% SDS–polyacrylamide gel electrophoresis.

Affinity Chromatography. Caldesmon was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the procedures outlined by Pharmacia. About 12 mg of caldesmon was linked to 3 mL of Sepharose. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and molecular weight determinations were carried out as described in one of our earlier publications (Mani & Kay, 1983). Protein concentrations were determined using $E^{1\%}_{1cm,278nm}$ values of 6.8 for caltropin (Mani & Kay, 1990), 3.8 for caldesmon (Bryan et al., 1989), and 7.0 for smooth muscle HMM (Okamoto & Sekine, 1978). Circular dichroism measurements were made on a J-720 spectropolarimeter (Jasco, Inc., Easton, MD) as described previously (Muni & Kay, 1992). HMM Ca^{2+}-ATPase activities were measured at room temperature. The solvent system used for the assay consisted of 10 mM Tris, pH 7.5, 0.5 M KCl, 1 mM $CaCl_2$, and 0.5 mM DTT. Each assay tube contained 0.05–0.1 mg of HMM. The reaction was initiated by adding Mg^{2+}-ATP to 2 mM and was terminated after 5 min of incubation by adding 5% trichloroacetic acid. The amount of P_i liberated was determined according to Itaya and Ui (1966) using the basic dye malachite green.

Fluorescence Studies. Fluorescence spectra were obtained with a Perkin-Elmer Model MPF-44 spectrofluorometer, and all measurements were made at 20 °C. The instrument was operated in a ratio mode simply to correct time-dependent lamp intensity fluctuation. Caldesmon was labeled with acrylodan following the procedure of Prendergast et al. (1983). Acrylodan was first dissolved in dimethylformamide. Caldesmon in 50 mM NaCl, 20 mM Tris (pH 7.5), 0.5 mM DTT, and 0.2 mM $CaCl_2$ was incubated with excess acrylodan at 4 °C for 18 h. After labeling, excess acrylodan was removed by passing the labeled caldesmon after centrifugation at 4 °C through a desalting column (Bio-Rad). The concentration of the label was determined from the absorbance at 380 nm using a molar extinction coefficient at 16 400 M^{-1} cm^{-1} (Prendergast, 1983). The ratio of label to protein was determined to be 1.7, suggesting that both of the sulfhydryl groups in caldesmon were labeled under these conditions. Labeled caldesmon was titrated with HMM by following the changes in the fluorescence intensity at 517 nm.

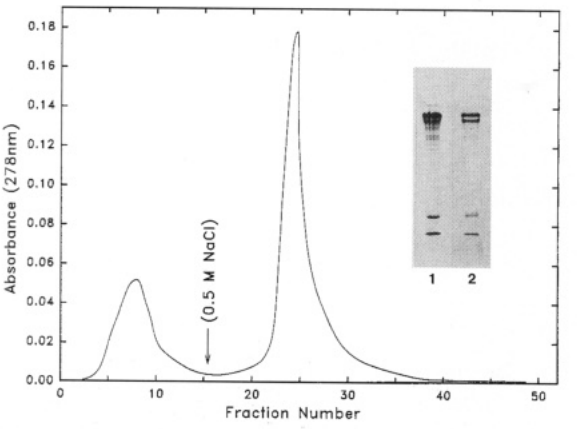

FIGURE 1: Caldesmon–sepharose affinity chromatography of heavy meromyosin. HMM (1.5 mg) was applied to a caldesmon–Sepharose column equilibrated with buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 40 mM NaCl, and 0.2 mM $CaCl_2$. The column was washed with the buffer, and at the point indicated by the arrow, the bound protein was eluted with the same buffer containing 0.5 M NaCl. The inset shows the SDS gel electrophoretic profile of control HMM (lane 1) and HMM eluted from the column with 0.5 M NaCl (lane 2).

Acrylamide Fluorescence Quenching Studies. The excitation wavelength used was 375 nm, at which the probe (acrylodan) has maximum absorption. In all instances, the initial A_{375} of the labeled caldesmon was ≤0.05; thus avoidance of the inner filter effect was assured. The fluorescence quenching was measured at the emission maximum (517 nm) of the labeled caldesmon and was initiated by adding small aliquots of 8 M acrylamide solution. The theory of acrylamide quenching and the mathematical treatment of the data are described by Lehrer and Leavis (1978).

RESULTS

Interaction of HMM with Caldesmon. Evidence for the direct binding of HMM to caldesmon was obtained using affinity chromatography (Figure 1). When HMM was applied to a caldesmon–Sepharose 4B column equilibrated with 20 mM Tris, pH 7.5, 40 mM NaCl, 1 mM DTT, and 0.2 mM $CaCl_2$, most of the protein was retained by the column. HMM that was bound was eluted by increasing the sodium chloride concentration to 0.5 M NaCl. An SDS gel electrophoretic pattern of the protein eluted with 0.5 M NaCl is shown in Figure 1 (inset). Also included in this figure is the gel profile obtained for the isolated HMM, prior to affinity chromatography, used as a control. On the basis of the peptide composition, as revealed by the gel profile, the bound protein was identified as HMM. In addition, ATPase measurements in the absence of actin were also carried out. Control HMM had a Ca^{2+}-activated ATPase of 110 nmol of P_i/min/mg at room temperature, while the protein that was bound to the caldesmon affinity column and subsequently eluted with 0.5 M NaCl had an activity of 120 nmol of P_i/min/mg at room temperature, suggesting that HMM had retained its full biological activity. HMM could also bind to a caldesmon–Sepharose column equilibrated with buffer containing 1 mM EGTA, suggesting that the interaction is not Ca^{2+}-dependent. The protein which did not bind to this affinity column was also identified as HMM on the basis of its SDS gel profile, and it corresponded to nearly 25% of the total protein applied. However, this protein peak had a lower ATPase activity (~80 nmol of P_i/min/mg at room temperature), and for this reason it may be effective in binding to this affinity column. The binding of HMM to the caldesmon column was also examined

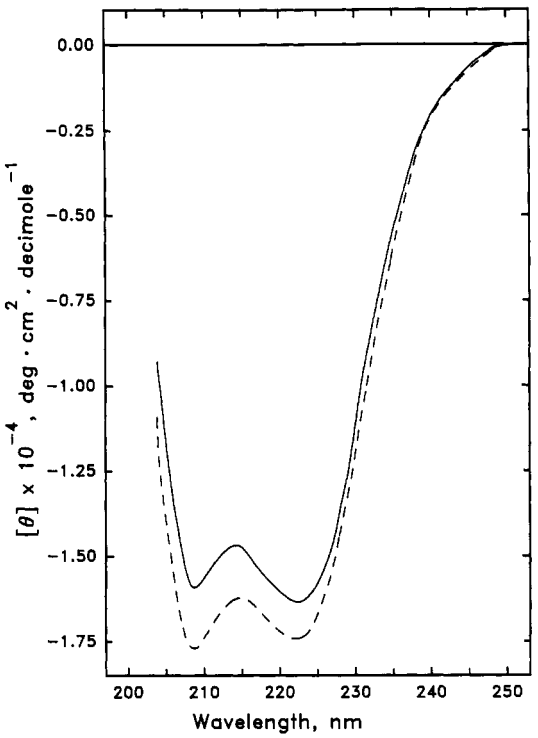

FIGURE 2: Far-ultraviolet circular dichroism spectrum for the caldesmon–HMM complex in 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.2 mM $CaCl_2$, and 40 mM NaCl (—) and the theoretical circular dichroism spectrum for an equimolar mixture of these two proteins (- - -).

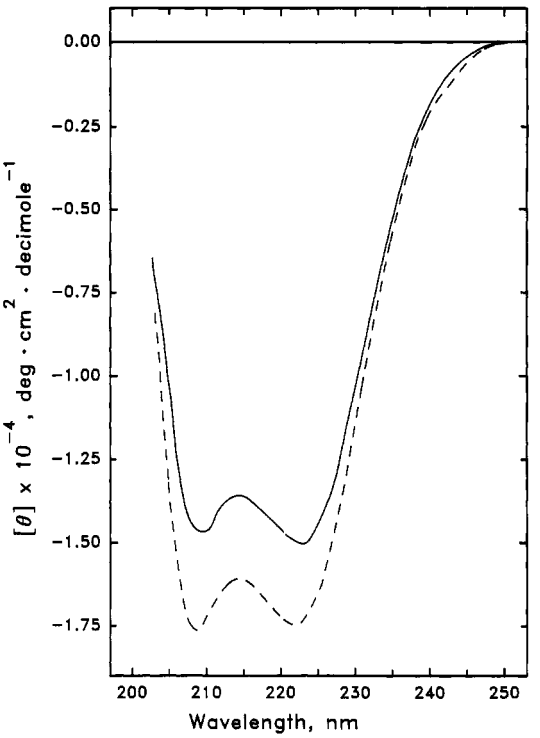

FIGURE 3: Far-ultraviolet circular dichroism spectrum for the caldesmon–caltropin–HMM ternary complex in 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.2 mM $CaCl_2$, and 40 mM NaCl (—) and the theoretical circular dichroism spectrum for a mixture of these three proteins (- - -).

in the presence of caltropin. For this experiment, 1 mg of HMM was first mixed with 0.1 mg of caltropin in buffer containing 20 mM Tris, pH 7.5, 40 mM NaCl, 1 mM DTT, and 0.2 mM $CaCl_2$. When this protein mixture was applied to the caldesmon column, both proteins (HMM and caltropin) were retained on this column and were eluted with 0.5 M NaCl. Hence, HMM can bind to caldesmon in the presence of caltropin, but its affinity for caldesmon was lowered significantly in the presence of caltropin (see below, Fluorescence Spectroscopy).

Circular Dichroism Studies. Figure 2 shows the far-ultraviolet circular dichroism spectrum for the HMM–caldesmon complex in the presence of 0.2 mM Ca^{2+}. HMM and caldesmon were mixed in a molar ratio of 0.5:1.0, respectively, since two caldesmon molecules can bind to a molecule of HMM (Hemric & Chalovich, 1990). CD measurements were also carried out using equimolar ratios. Molecular weights of 352 000 and 87 000 were used for HMM and caldesmon, respectively. Figure 2 also includes a theoretical curve for HMM–caldesmon in the presence of Ca^{2+} calculated from the ellipticity values of HMM and caldesmon alone using a molar ratio of 0.5:1.0, respectively. The experimentally observed ellipticity values deviate from the theoretical values particularly in the 222- and 207-nm wavelength regions, suggesting that the interaction has produced a conformational change. For instance, at 222 nm the difference between the observed and the theoretical ellipticity values was nearly 1300 deg cm^2 $dmol^{-1}$, while the experimental error in these measurements is only ± 300 deg cm^2 $dmol^{-1}$, thus clearly indicating that the interaction has produced a conformational change. Analysis of the CD data according to the Chen et al. (1974) method indicated a decrease in the apparent α-helical content upon complex formation. HMM–caldesmon complex had an apparent α-helical content of nearly 52% compared to a value of 58% for the calculated CD spectrum of the two proteins. When the two proteins were mixed in an equimolar ratio, the observed conformational change was of a smaller magnitude since the experimentally observed ellipticity at 222 nm deviated from the theoretical value by only 800 deg cm^2 $dmol^{-1}$. Interaction between HMM and caldesmon was also monitored in the absence of Ca^{2+}, and this was achieved by replacing 0.2 mM Ca^{2+} in the solvent system by 1 mM EGTA. Interaction between HMM and caldesmon in the absence of Ca^{2+} also produced a conformational change, and the observed change was comparable to the Ca^{2+} state, thereby implying that these two proteins can interact even in the absence of calcium ions; this finding is in line with our affinity column results.

A far-UV CD spectrum of the caltropin–caldesmon–HMM ternary complex is shown in Figure 3. Caltropin, caldesmon, and HMM were initially mixed in molar ratios of 1:1:0.5, respectively, using the subunit molecular weight of 11 000 for caltropin and the molecular weights for caldesmon and HMM as stated earlier in reference to Figure 2. Formation of the ternary complex also produced a conformational change since the observed ellipticity at 222 nm differed from the theoretical value by 2300 deg cm^2 $dmol^{-1}$. However, when the three proteins were mixed in the absence of Ca^{2+}, i.e., in 1 mM EGTA, there were no evidence for any ternary complex formation since the observed ellipticity at 222 nm was in excellent agreement with the theoretical value. Alternatively, one can interpret the result as follows. Binding of HMM to caldesmon decreases the ellipticity value around the 222-nm region. Further binding of caltropin to the binary complex produces a countereffect which results in increasing the ellipticity value at 222 nm in the absence of Ca^{2+}, with the result that there is no resultant change in the ellipticity value at 222 nm. However, our earlier work (Mani et al., 1992) had shown that caltropin can interact with caldesmon only in the presence of Ca^{2+}; hence, it is unlikely that the latter

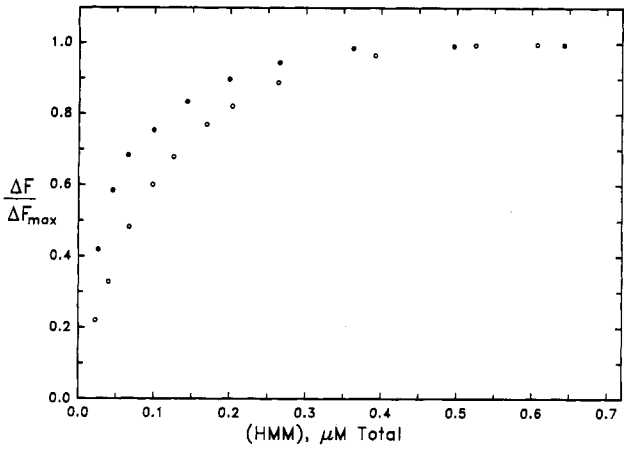

FIGURE 4: Influence of HMM on acrylodan caldesmon (●) and acrylodan caldesmon–caltropin fluorescence (○). The initial caldesmon concentration was 1.2×10^{-7} M. Measurements were carried out in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, 0.2 mM $CaCl_2$, and 0.5 mM DTT at 20 °C. Relative change in fluorescence intensities ($\Delta F/\Delta F_{max}$) at 517 nm are plotted as a function of HMM concentration. The extinction wavelength was 375 nm.

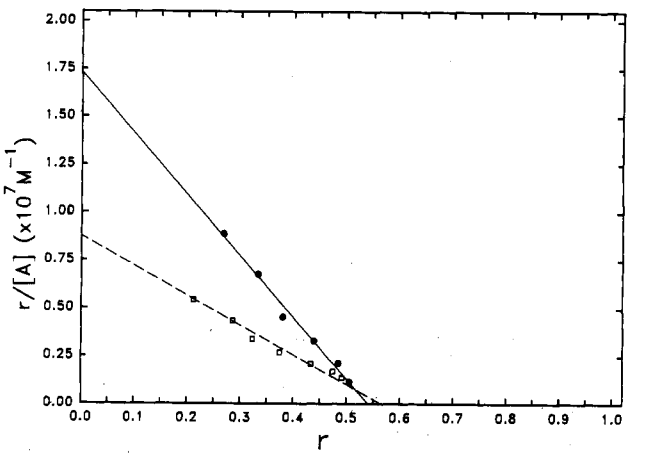

FIGURE 5: Scatchard plot of HMM binding to acrylodan caldesmon (●) and acrylodan caldesmon–caltropin (□) with [HMM] corrected to [free HMM] using a 2:1 stoichiometry for caldesmon to HMM. Bound HMM was determined from the change in fluorescence intensity, which is directly proportional to binding. γ is the number of moles of ligand bound to 1 mole of macromolecules; it is defined as $\gamma = [A]_{bound}/[P]_{total}$. The conditions were the same as described in the caption of Figure 4.

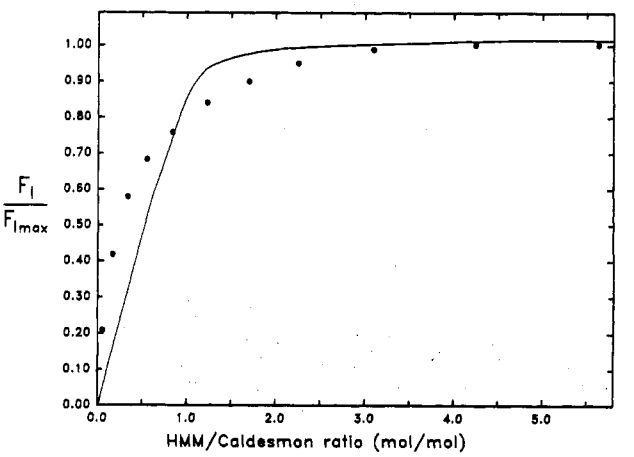

FIGURE 6: Effect of HMM on the fluorescence of acrylodan caldesmon using the conditions described in Figure 4. The smooth curve through the data points was calculated for the binding of 1 mol of HMM/mol of caldesmon.

phenomenon operates, and for this reason we believe the observed effect is evidence of no ternary complex formation in the absence of Ca^{2+}. The formation of this ternary complex in the presence of Ca^{2+} was explored in further detail. In our experiment, HMM was added to the binary complex of caltropin and caldesmon, and in another set of experiments, caltropin was added to the HMM–caldesmon binary complex. The far-UV CD spectra generated for these two sets of experiments were virtually identical, suggesting that the order of addition is nor important in ternary complex formation. This would imply that the binding sites for caltropin and HMM on caldesmon must be different and as a result there is no competition. This observation is consistent with our affinity column results since HMM can bind to a caldesmon column in the presence of Ca^{2+}-caltropin.

Fluorescence Spectroscopy. Protein–protein interaction can often be studied using fluorescence spectroscopy. Since both caldesmon and HMM have tyrosine and tryptophan residues, one cannot easily study the interaction between these two proteins using the intrinsic fluorescence of tyrosine and tryptophan residues. For this reason we decided to use acrylodan, a sulfhydryl probe, to label caldesmon. Under the experimental conditions used, the ratio of label to caldesmon obtained was 1.7, suggesting that both sulfhydryl groups present in caldesmon were labeled with acrylodan. When the labeled caldesmon was excited at 375 nm, the emission maximum occurred at 517 nm both in the presence and in the absence of calcium. Addition of HMM resulted in a nearly 20% decrease in fluorescence intensity with no significant change in the emission maximum.

We carried out a fluorescence titration to establish the stoichiometry and the affinity with which HMM bound to labeled caldesmon. Analysis of the titration curve (Figure 4) indicated that more than 70% of the fluorescence change was completed by the time 0.5 mol of HMM was added per mol of caldesmon. At equimolar ratio, the observed fluorescence change was greater than 80%. If one assumes that the observed fluorescence change is the result of HMM binding to caldesmon, then one can calculate the amount of HMM bound at any given point in the titration curve. Knowing the amount of HMM added, one can deduce the amount of the free HMM that is present. When Scatchard analysis was carried out, with the amounts of the bound and free HMM and the caldesmon concentration used for the titration known, a best fit was obtained when the ratio of caldesmon to HMM was 2:1, since nearly 0.55 mol of HMM binds to 1 mol of caldesmon (Figure 5). This indicates the existence of two caldesmon binding sites on HMM, in agreement with an earlier observation made by Hemric and Chalovich (1990). Moreover, analysis of the titration curve by curve fitting, as explained in our earlier paper (Mani et al., 1992), did not give a good fit when the molar ratio of HMM to caldesmon was assumed to be 1:1 (Figure 6). From the slope term of the Scatchard plot a K_a value of $(4.5 \pm 0.5) \times 10^7$ M^{-1} was obtained from four sets of titrations, suggesting that HMM binds to caldesmon with high affinity. Also shown in the figure is the titration of HMM with caldesmon–caltropin complex (Figure 5). HMM binds to this binary complex with a lower affinity since the observed increases in fluorescence intensity in this instance were only around 50% and 65% when the HMM to caldesmon ratios were 0.5:1 and 1:1, respectively (Figure 4). The observed K_a value was $(1.5 \pm 0.5) \times 10^7$ M^{-1}. The presence of caltropin has lowered the affinity of HMM for caldesmon by nearly 3-fold. The changes in fluorescence intensity versus HMM concentration plotted as the double-inverse plots are shown in Figure 7. The affinities of HMM for caldesmon

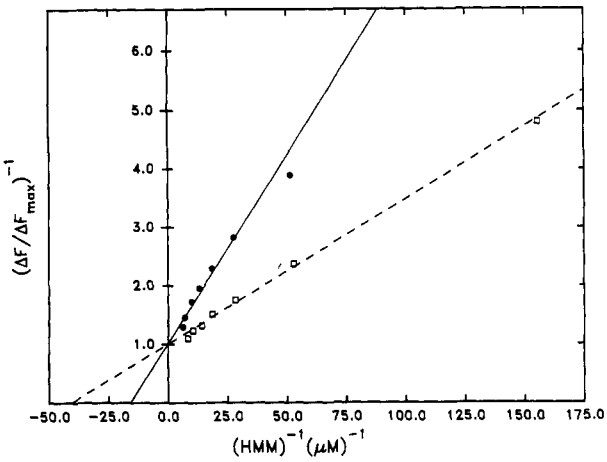

FIGURE 7: Double-inverse plots of changes in fluorescence of acrylodan caldesmon (□) and acrylodan caldesmon–caltropin (●) as a function of HMM concentration. The concentration of acrylodan caldesmon was kept constant at 1.2×10^{-7} M in 20 mM Tris-HCl (pH 7.5), 40 mM NaCl, 0.5 mM DTT, and 0.2 mM $CaCl_2$ at 20 °C. The excitation wavelength was 375 nm, and the monitored emission wavelength was 517 nm.

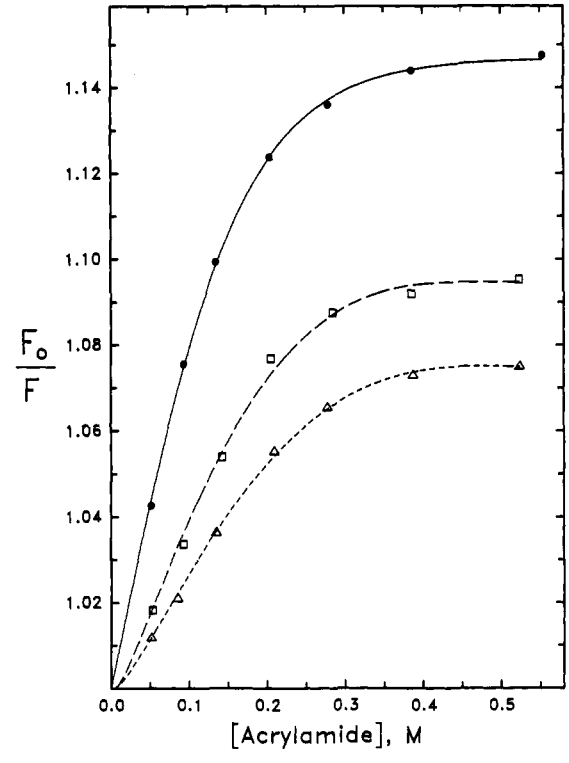

FIGURE 8: Steady-state acrylamide quenching plots for acrylodan caldesmon (●), acrylodan caldesmon–HMM complex (△), and the ternary complex of acrylodan caldesmon–HMM–caltropin (□). Excitation and emission wavelengths were 375 and 517 nm, respectively. The solvent system used was the same as in Figure 4.

and the caldesmon–caltropin complex determined from the abscissa intercepts of the double-inverse plots were 4.0×10^7 and 1.5×10^7 M^{-1}, respectively. Since the double-inverse plots are linear, one may conclude that both the sites on HMM bind caldesmon with similar affinity.

Acrylamide Quenching Studies. Figure 8 shows the effect of the acrylamide quencher on the fluorescence intensity of acrylodan-labeled caldesmon, labeled caldesmon–HMM complex, and labeled caldesmon–caltropin–HMM ternary complex. The linear portion of the plots at low acrylamide concentration gave Stern–Volmer constants (K_{SV}) of 0.9, 0.4, and 0.28 M^{-1} for labeled caldesmon, ternary complex, and caldesmon–HMM binary complex, respectively. The values of the Stern–Volmer constants obtained suggest that the probe is least accessible to acrylamide in the binary complex. At high acrylamide concentration the effect of quenching seems to level off, and this may be considered as an example of downward curvature from linearity due to reduced efficiency of quenching at high acrylamide concentration. This downward curvature has also been observed with other protein systems (Eftink & Ghiron, 1976; Mani & Kay, 1985).

DISCUSSION

The fact that caldesmon can bind tightly to myosin as well as to actin suggests a novel mode of interaction between thick and thin filaments in smooth muscle. Caldesmon binds to actin and tropomyosin tightly with affinities near 10^7 and 10^6 M^{-1}, respectively, and these interactions have been extensively studied (Velaz et al., 1989; Graceffa & Jancso, 1991; Horiuchi & Chacko, 1988; Crosbie et al., 1991, 1992). The actin binding region(s) are largely confined to a C-terminal 35-kDa fragment (Szpacenko & Dabrowska, 1986; Fujii et al., 1987), while the myosin binding activity is localized to an N-terminal chymotryptic fragment of caldesmon (Velaz et al., 1989). Caldesmon also competes with myosin fragments for the binding to actin in rigor (Hemric & Chalovich, 1988), and this indicates the existence of at least a partial overlap in their actin binding sites and could form the basis of the regulatory function of caldesmon. Chalovich et al. (1987) have proposed a model in which caldesmon acts as a competitive inhibitor of the binding of myosin to actin. Support for this model comes from the fact that the C-terminal end of caldesmon, which does not bind to myosin, is able to inhibit the hydrolysis of ATP in parallel with inhibition of binding of smooth muscle HMM–ATP to actin (Velaz et al., 1990; Horinchi & Chacko, 1989). Hemric and Chalovich (1990) studied the binding of [^{14}C]iodoacetamide-labeled caldesmon to smooth muscle myosin filaments by a low-speed sedimentation assay. In their assay nearly 26% of the labeled caldesmon was inactive, and this inactive caldesmon did not bind to myosin even at saturating myosin concentrations; according to the authors it affected the constant (K_a) for caldesmon binding to myosin even though it had little impact on the stoichiometry. Hence, the K_a values of 10^6 and 10^5 M^{-1} reported for caldesmon binding to myosin and HMM could conceivably be significant lower than the real affinities. The stoichiometry established was 2.5 mol of caldesmon per mol of myosin in the absence of ATP and 1:1 in the presence of ATP. This observed effect of ATP on the binding of caldesmon to smooth muscle myosin provides further evidence for the specificity of the interaction between caldesmon and myosin; i.e., the interaction between caldesmon and myosin is sensitive to the conformation of both proteins.

In the present study we have provided direct evidence for interaction between HMM and caldesmon since HMM was retained on a caldesmon–Sepharose affinity column. When HMM and caltropin in the presence of Ca^{2+} were applied to a caldesmon affinity column, both proteins were retained, suggesting that these two proteins bind at different sites on caldesmon. Even though caldesmon can bind to HMM, it had no significant effect on the Ca^{2+}-activated ATPase activity of HMM (0.5 M KCl) in the absence of actin. In this regard, our finding is similar to an earlier observation made by Chalovich et al. (1990). This would imply that the caldesmon binding site on myosin must be away from the head region containing the ATPase activity. In fact, caldesmon is known to bind at the rod portion of myosin, i.e., in the S_2 region (Hemric & Chalovich, 1990).

Spectroscopic studies have proved very useful in our hands for studying protein–protein interactions. Far-UV CD measurements indicated a conformational change as a result of an interaction between caldesmon and HMM, since the observed ellipticity values differed from the theoretical ones and this interaction was not Ca^{2+}-dependent. However, when HMM was added to caldesmon in the presence of caltropin, the interaction was Ca^{2+}-dependent. CD studies also suggested that HMM and caltropin probably bind at different sites on caldesmon since the order of addition was not important for ternary complex formation.

Since sulfhydryl groups in chicken gizzard caldesmon can be modified without the protein losing its ability to bind myosin (Hemric & Chalovich, 1990), we decided to label both the sulfhydryl groups in caldesmon with acrylodan. Addition of smooth muscle HMM to labeled caldesmon resulted in a 20% decrease in fluorescence intensity with no significant change in the emission maximum. Titration of HMM with labeled caldesmon indicated that it binds with high affinity, and the K_a value obtained was $(4.5 \pm 0.5) \times 10^7$ M^{-1}. This is at least an order of magnitude higher than the K_a of 10^6 M^{-1} reported for myosin using a low-speed sedimentation assay (Hemric & Chalovich, 1990), and the problem in determining K_a using this approach was discussed earlier. In the present study, we are monitoring the fluorescence intensity of the label, and the observed change in fluorescence intensity is due to the binding of HMM at or near the label(s). Hence, the K_a value determined should represent its affinity for HMM. It also became clear from this study that this interaction between caldesmon and HMM is not Ca^{2+}-dependent, in agreement with ATPase activity studies; i.e., caldesmon can inhibit actin-activated myosin ATPase even in the absence of Ca^{2+}. Acrylamide quenching studies indicated that the label was least accessible to acrylamide in the caldesmon–HMM complex. From this result, one may conclude that HMM binds to caldesmon at a region near the label (Cys-153, since HMM is known to bind at the N-terminal end of caldesmon) and thus shields the label from collisions with quenchers in the medium.

In the present study, we have shown that caldesmon binds to HMM with high affinity and that this interaction is weakened in the presence of caltropin (Ca^{2+}). Caltropin, which is very potent compared to calmodulin in reversing the inhibitory effect of caldesmon in the presence of calcium (Mani et al., 1992), is also able to modulate the interaction between caldesmon and HMM in a Ca^{2+}-dependent manner. Thus caltropin could conceivably by the calcium factor involved in regulating caldesmon in smooth muscle. On the basis of our results the caltropin effect could be summarized as follows. As caldesmon can bind to both actin and myosin with high affinity, it is able to inhibit the actin-activated myosin ATPase activity by interfering in the actin–myosin interaction. In the presence of Ca^{2+}-caltropin, the affinity of caldesmon for myosin is lowered nearly 3-fold with the result that myosin is able to interact effectively with actin, thereby restoring the actin-activated myosin ATPase activity. Alternatively, caldesmon in the absence of caltropin acts as a competitive inhibitor of myosin binding to actin, resulting in a lowered ATPase level since caldesmon can bind to actin at or near the myosin binding site, thereby blocking myosin from binding to actin. Caldesmon, which binds to caltropin in the presence of Ca^{2+} with high affinity [$K_a \approx 10^7$ M^{-1} (Mani et al., 1992)], undergoes a conformational change, with the result that it no longer is able to compete with myosin for binding to actin. In other words, caldesmon in the presence of caltropin has a lower affinity for actin. In this context, it should be noted that Ca^{2+}-calmodulin is known to weaken the interaction of caldesmon with actin near the myosin binding site of actin (Graceffa & Jansco, 1991), thus allowing myosin to bind to actin, resulting in the recovery of ATPase activity. Future studies involving the interaction of caldesmon with actin in the presence of caltropin should shed more light on our understanding of the role of caltropin in caldesmon regulation in smooth muscle.

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