Arrangement of the COOH-Terminal and NH_2-Terminal Domains of Caldesmon Bound to Actin†

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ABSTRACT: Smooth muscle caldesmon is a single polypeptide chain with its NH_2- and COOH-terminal domains separated by a long α-helix. Caldesmon was labeled at either Cys-153 in the NH_2 domain or Cys-580 in the COOH domain with a variety of fluorescence probes. Fluorescence intensity, peak position, and polarization of probes on Cys-580 were very sensitive to the binding to actin (with or without tropomyosin), whereas for probes on Cys-153, there was a lack of response, in reconstituted or native actin thin filaments. From fluorescence resonance energy transfer from donor labels on either caldesmon cysteine to acceptor labels on Cys-374 of actin, the distance between the donor and acceptor was estimated to be 27 Å for the donor at Cys-580 and 65−80 Å for the donor at Cys-153. These findings were the same for caldesmon prepared with or without heat treatment and with striated or smooth muscle actin. These results, together with previous knowledge that COOH-terminal fragments of caldesmon bind to actin whereas NH_2-terminal fragments do not, indicate that, while the COOH domain of caldesmon is bound to actin, the NH_2 domain is largely dissociated. Fluorescence quenching studies showed that actin binding to caldesmon greatly decreased the accessibility of probes at caldesmon Cys-580 to the quencher, whereas for probes at Cys-153, actin afforded much less, but significant, protection from quenching. Consequently, it appears that, although the NH_2 domain is mostly dissociated, it spends some time in the vicinity of actin, through either a weak interaction with actin or collisions with actin and/or because of restricted flexibility which constrains the NH_2 domain to be close to the actin filament. Since the NH_2 domain of caldesmon binds to the neck region of myosin, a dissociated NH_2 domain may account for caldesmon's ability to link myosin and actin filaments.

Smooth muscle contraction is primarily regulated on the thick filament by myosin phosphorylation (Kamm et al., 1985), although physiological evidence indicates that this mechanism cannot fully explain regulation (Kamm & Stull, 1989; Gerthoffer, 1991). It appears that there is additional regulation on the thin filament involving the actin binding protein caldesmon, although its role is not clearly understood (Allen & Walsh, 1994). Chicken gizzard caldesmon is a 756-residue polypeptide chain forming an elongated, thin molecule with a COOH-terminal domain, which binds to actin and inhibits actomyosin ATPase activity, an NH_2-terminal domain, which contains a binding site for the "neck region" of myosin, and a central domain consisting of a long α-helix (Wang et al., 1991; Graceffa & Jancso, 1993) separating the other two domains [reviewed in Gusev et al. (1991), Sobue and Sellers (1991), and Marston and Redwood (1991)]. Caldesmon's inhibition of actomyosin ATPase activity, which can be reversed by Ca^{2+}−calmodulin [reviewed in Gusev et al. (1991), Sobue and Sellers (1991), and Marston and Redwood (1991)] or Ca^{2+}−caltropin (Mani et al., 1992) binding to caldesmon, may play a role in regulation (Allen & Walsh, 1994).

Since the NH_2 and COOH domains of caldesmon bind to myosin and actin, respectively, it has also been proposed that a simultaneous binding of caldesmon to myosin in the thick filament and actin in the thin filament could link the filaments and possibly (1) enhance an interaction between myosin and actin (Haeberle et al., 1992), (2) promote the assembly of myosin filaments in the vicinity of actin filaments (Katayama et al., 1995), or (3) account for the passive tension of the latch state (Hemric & Chalovich, 1988; Ikebe & Reardon, 1988; Sutherland & Walsh, 1989; Marston, 1989), the energy-economic state of smooth muscle which exhibits full tension at low levels of ATP hydrolysis and myosin phosphorylation (Dillon et al., 1981). There is in vitro evidence that thick and thin filaments can be linked by caldesmon (Haeberle et al., 1992; Marston et al., 1992; Hemric et al., 1994; Katayama et al., 1995). However, whether this link can support the mechanical load of the latch state is controversial (Haeberle et al., 1992; Pfitzer et al., 1993; Horiuchi & Chacko, 1995; Fraser & Marston, 1995).

An understanding of the arrangement of the COOH- and NH_2-terminal domains of actin-bound caldesmon will be helpful in uncovering the function of caldesmon. That COOH-terminal fragments of caldesmon bind to actin whereas NH_2-terminal fragments do not is well-established. (Szpacenko & Dabrowska, 1986; Fujii et al., 1987; Katayama et al., 1989; Hayashi et al., 1991; Redwood & Marston, 1993). However, one cannot conclude from fragment studies whether or not the NH_2 domain of intact caldesmon binds to actin, since proteolysis might have damaged the actin binding of the NH_2 domain, or that, although the actin binding of the NH_2 domain is inherently weak, it is necessary to have the COOH domain of intact caldesmon bound to actin in order to increase NH_2 domain binding by increasing

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its effective concentration in the vicinity of the actin filament. Several micrcoscopy studies have been carried out on intact caldesmon in order to address this question. Some of these studies do not observe projections of caldesmon from native (Moody et al., 1990; Popp & Holmes, 1992; Mabuchi et al., 1993) or reconstituted (Moody et al., 1990; Popp & Holmes, 1992) actin thin filaments and have interpreted this as evidence that the entire length of caldesmon, including its NH_2 domain, is bound to actin. However, the microscopy studies are controversial since one study (Mabuchi et al., 1993) finds projections in reconstituted filaments but not in native filaments, while another study (Katayama & Ikebe, 1995) finds caldesmon projections in both native and reconstituted filaments.

It is the object of the present work to investigate the possible actin interaction of the NH_2 domain of intact caldesmon in solution, without the use of fixing procedures necessary for microscopy studies. In our previous study (Graceffa, 1995), the fluoresecence intensity and polarization of a rhodamine probe specifically attached to either Cys-580 or Cys-153 of caldesmon, together with experiments on the cross-linking of the Cys-580 and Cys-153 regions of caldesmon to actin, suggested that the NH_2 domain is dissociated from reconstituted actin filaments while the COOH domain is strongly bound. We have extended the fluorescence study, in the present work, by using a variety of additional probes, by employing the additional fluorescence techniques of fluorescence quenching and fluorescence resonance energy transfer, and by extending the study to native actin filaments. This investigation provides futher evidence for a largely dissociated NH_2 domain of caldesmon bound to actin thin filaments.

EXPERIMENTAL PROCEDURES

The experimental procedures and preparations used in this study are described in the previous work (Graceffa, 1995) unless stated otherwise. The preparation of caldesmon with a heat treatment step was carried out as described (Graceffa & Jancso, 1991) with the addition of calmodulin affinity chromatography (Sobue et al., 1981). Native thin filaments were prepared from fresh chicken gizzard as reported (Vibert et al., 1993) with additional proteolytic inhibitors (i.e. 0.25 mM PMSF, 0.75 mM benzamidine, and 0.2 μg/mL pepstatin A) used throughout the preparation. Furthermore, the concentration of casein, used as a protease sink (Vibert et al., 1993), was reduced by $^1/_2$ in order to minimize casein in the final native thin filament preparation.

Fluorescence Labeling of Caldesmon. Just prior to labeling, caldesmon was exhaustively dialyzed vs 40 mM NaCl, 5 mM Mops,[1] and 0.2 mM EDTA at pH 7.5 in order to remove dithiothreitol which was present in stored caldesmon to maintain cysteine residues in the reduced state. Caldesmon was specifically labeled at either Cys-153 or Cys-580 with sulfhydryl-specific fluorescent probes as reported previously (Graceffa, 1995) and described briefly as follows. The labeling of Cys-580 was performed with porcine stomach caldesmon which contains a single cysteine equivalent to position 580 of chicken gizzard caldesmon (Graceffa et al., 1993). The labeling was carried out as described previously (Graceffa, 1995) except that a lower probe to caldesmon molar ratio of 3 (instead of 5) was used and the labeling proceeded for 3 h on ice before adding excess dithiothreitol. Labeling of Cys-153 was performed with chicken gizzard caldesmon which contains cysteines at positions 580 and 153. Cys-580 was first disulfide cross-linked to Cys-374 of 5,5′-dithiobis(2-nitrobenzoic acid)-modified actin whereupon Cys-153 was free to be modified (Graceffa & Jancso, 1991; Graceffa, 1995). The disulfide bond was then cleaved with dithiothreitol and the Cys-153-labeled caldesmon separated from the actin. The probes used, all from Molecular Probes, were 6-acryloyl-2-(dimethylamino)naphthalene (AC or acrylodan), 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM or coumarin), 5-[[[(2-iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS), *N*-(1-pyrene)maleimide (PM or pyrene), and 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS). For the PM label, the final dialysis was performed exhaustively over a period of several days to remove any PM noncovalently bound to the protein.

The degree of labeling was estimated from the caldesmon concentration, measured by the Lowry assay (Graceffa & Jancso, 1993), and the probe concentration, using the following probe extinction coefficients ϵ_{360nm}(AC) = 12 900 (Prendergast et al., 1983), ϵ_{387nm}(CPM) = 30 000 (Zot et al., 1990), ϵ_{337nm}(IAEDANS) = 6100 (Hudson & Weber, 1973), ϵ_{343nm}(PM) = 23 000 (Graceffa & Lehrer, 1980), and ϵ_{320nm}(MIANS) = 22 000 (Forgac, 1980). The degree of labeling for Cys-580-labeled caldesmon was between 0.6 and 1.1 mol of label per mole of caldesmon, consistent with the specific labeling of the single cysteine of the porcine caldesmon. The fact that the fluorescence decay of the IAEDANS-labeled (porcine) caldesmon could be fit with a single lifetime component (see below) is also consistent with the homogeneous labeling of the single cysteine of this caldesmon. The degree of labeling for Cys-153-labeled chicken caldesmon was between 0.3 and 0.8 mol of label per mole of caldesmon. In order to determine the specificity of the Cys-153 labeling, the labeled caldesmon was digested with thrombin, according to Mornet et al. (1988), which cleaves chicken gizzard caldesmon at position 483 (Mornet et al., 1988; Leszyk et al., 1989) resulting in a large NH_2-terminal fragment containing Cys-153 and a small COOH-terminal fragment containing Cys-580. Only the NH_2-terminal fragment was fluorescent, as shown previously for Cys-153-labeled caldesmon (Graceffa & Jancso, 1991), consistent with the specific labeling of Cys-153. Further evidence for specific labeling of Cys-153 was the fact that the pyrene-labeled protein showed no excimer fluorescence (data not shown) which exists for gizzard caldesmon labeled at both Cys-153 and Cys-580 (Horiuchi & Chacko, 1988).

Labeling Actin Cys-374 with Fluorescence Acceptors. F-actin, in F-buffer (40 mM NaCl, 2 mM $MgCl_2$, 0.01% NaN_3, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 2 mM Mops at pH7.5), was labeled with fluorescein maleimide (FM) (Molecular Probes) or *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide (DDPM) (Aldrich) at a label/actin molar ratio

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; AC or acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; CPM or coumarin, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; IAEDANS, 5-[[[(2-iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; PM or pyrene, *N*-(1-pyrene)maleimide; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; FM, fluorescein maleimide; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; CaD, caldesmon; Tm, tropomyosin; FRET, fluorescence resonance energy transfer.

of 2 at 0 °C overnight, followed by the addition of 2 mM dithiothreitol to react with excess label, and then dialyzed against F-buffer. It has been shown previously that the FM (Konno & Morales, 1985) and DDPM (Miki & Mihashi, 1978) labels react specifically with Cys-374, the most reactive cysteine of actin. The degree of labeling was determined by using the following probe extinction coefficients (M^{-1} cm^{-1}): ϵ_{494nm}(FM) = 52 000 (Wolff & Lai, 1990) and ϵ_{440nm}(DDPM) = 3000 (Gold & Segal, 1964). The concentration of labeled actin was determined by the Lowry assay using unlabeled actin as a standard. The labeling ratios were between 0.9 and 1.1, consistent with the specific labeling of Cys-374.

Fluorescence emission and polarization spectra were recorded as described previously (Graceffa, 1995). All measurements were performed at 20 °C in a buffer containing 40 mM NaCl, 2 mM $MgCl_2$, and 5 mM Mops at pH 7.5 with caldesmon at about 0.5 μM, actin at an actin/caldesmon molar ratio of 20, and Tm at an actin/Tm molar ratio of 7. The excitation wavelength for each probe was as follows: AC, 385 nm; CPM, 396 nm; IAEDANS, 340 nm; MIANS, 325 nm; and PM, 342 nm. For PM and MIANS spectra, a buffer spectrum was always subtracted since a Raman scattering peak overlapped the fluorescence peak.

Fluorescence resonance energy transfer, between a fluorescence donor specifically attached to either cysteine of caldesmon and an acceptor attached to actin, was measured by the decrease in the fluorescence lifetime and/or the steady-state fluorescence intensity of the donor [Stryer (1978), Wu and Brand (1994), and Selvin (1995) for reviews]. Steady-state fluorescence was measured with a Spex Fluorolog 2/2/2 photon-counting fluorometer with the sample cell thermostated at 20 °C and the excitation wavelength of the donor as described above. Fluorescence lifetime decay, at 20 °C, was obtained from a modified ORTEC 9200 nanosecond fluorometer and analyzed by the method of moments, as described by Tao and Cho (1979). Buffer and protein concentrations were as described above for fluorescence spectral measurements.

The donor/acceptor pairs used and their respective R_o's (the distance of 50% energy transfer efficiency) measured for other systems were as follows: CPM/FM (50−52 Å) (Odom et al., 1984; Snyder & Hammes, 1985; Richter et al., 1985) and IAEDANS/DDPM (27−29 Å) (Dalbey et al., 1983; Wang et al., 1992). The R_o's for the systems under present investigation were determined to have the following values: IAEDANS(CaD−Cys-580)/DDPM(actin−Cys-374), 27 Å; CPM(CaD−Cys-580)/FM(actin−Cys-374), 48 Å; and CPM(CaD−Cys-153/FM(actin−Cys-374), 49 Å. These values were determined from the equation

$$R_o = (8.79 \times 10^{-5} J Q_D n^{-4} \kappa^2)^{1/6}$$

where J is the normalized spectral overlap of the donor (on CaD) emission in the presence of unlabeled actin and the acceptor (on actin) absorption, Q_D is the quantum yield for the donor (on CaD) emission in the presence of unlabeled actin, n is the index of refraction taken to be 1.33, and κ^2 is the orientation factor assumed to have a value of $^2/_3$ [reviewed in Stryer (1978), Wu and Brand (1994), and Selvin (1995)]. The quantum yield was determined (Takashi, 1979) from the integrated fluorescence intensity compared to that of quinine sulfate in 0.1 N H_2SO_4 which has a quantum yield of 0.70 (Scott et al., 1970). Corrected fluorescence spectra were used in the determination of the quantum yield and the spectral overlap parameter J.

The distance between the donor and acceptor, r, was calculated from the equation

$$r = R_o(E^{-1} - 1)^{1/6}$$

where the energy transfer effeciency $E = 1 - f/f_o$, where f_o is the steady-state fluorescence (or fluorescence lifetime) in the presence of unlabeled actin (with or without Tm) and f is the fluorescence in the presence of acceptor-labeled actin (with or without Tm). The steady-state fluorescence f was corrected for any fluorescence from labeled actin, which was necessary for FM-labeled actin. Corrections, albeit small ones, were also made to the steady-state fluorescence for trivial absorption of the excitation and emission light due to absorption by labeled actin.

Fluorescence quenching by acrylamide (Eftink & Ghiron, 1976) was performed by measuring steady-state fluorescence of labeled caldesmon as a function of the addition of aliquots of 5 M acrylamide. Measurements were made in the presence and absence of actin (with or without Tm) under the solvent conditions and protein concentrations described above. The data was treated according to the Stern−Volmer equation:

$$F_o/F = 1 + k_q \tau_o [Q]$$

where F_o and F are the fluorescence intensities at the peak wavelength in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, τ_o is the fluorescence lifetime in the absence of quencher, and [Q] is the concentration of the quencher. $K = k_q\tau_o$ is the Stern−Volmer quenching constant which is obtained from the slope of a plot of F_o/F vs [Q] (Lakowicz, 1983). A small correction to F was necessary due to dilution by the added quencher. A measure of the accessibility of the fluorescence probe to the quencher is given by $k_q = K/\tau_o$. A relative value of k_q for a fluorophore under different conditions can be obtained using the relationship $\tau_o = kF_o^I$, where k is a constant for a particular fluorophore and F_o^I is the integrated fluorescence intensity of the fluorophore in the absence of quencher.

RESULTS

In the results discussed below, actin refers to that from rabbit skeletal muscle and caldesmon is prepared with a heat treatment step, unless otherwise stated, and the source of tropomyosin is always chicken gizzard smooth muscle. Native caldesmon refers to chicken gizzard caldesmon prepared without a heat treatment step. The actin/caldesmon molar ratio used is 20/1 in order to minimize any possible interference between caldesmon molecules on the actin filament which might reduce the interaction between the caldesmon NH_2 domain and actin. The actin/caldesmon molar ratio in native thin filaments has been reported to be 16/1 for actin filaments containing caldesmon (Lehman et al., 1989), although much lower values were found in reconstituted thin filaments saturated with caldesmon [see the discussion of Graceffa and Jancso (1991)].

Fluoresence Intensity, Peak Wavelength, and Polarization. In a previous work, we found that the fluorescence intensity

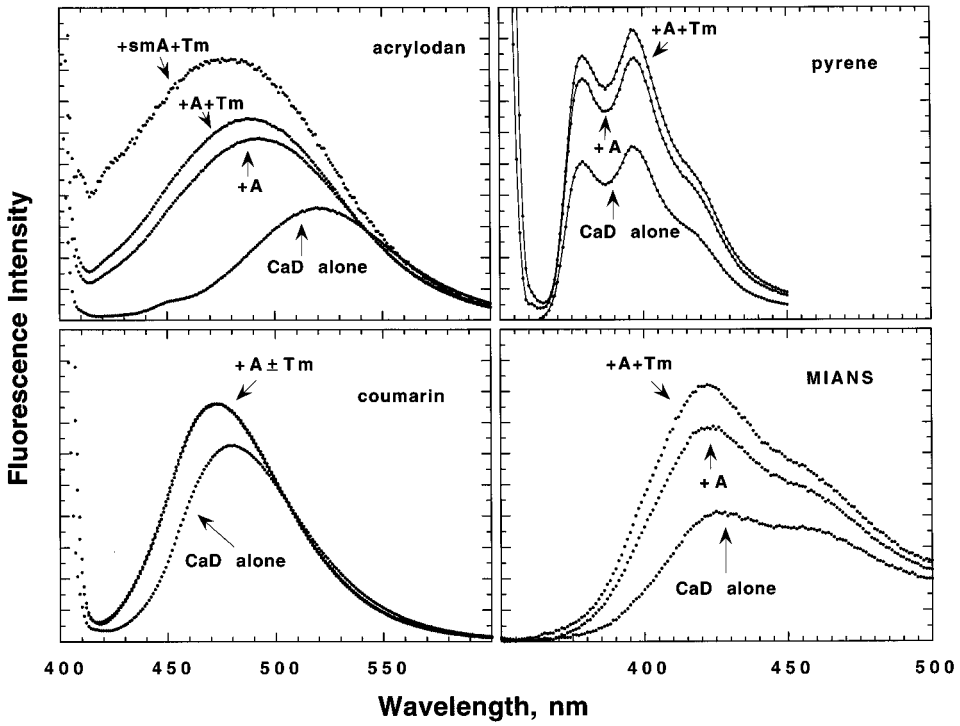

FIGURE 1: Fluorescence spectra of Cys-580-labeled CaD with or without actin and with or without tropomyosin (Tm). A = skeletal actin; smA = smooth actin. CaD is heat-treated. λ_{ex} values of probes are as follows: acrylodan, 385 nm; pyrene, 342 nm; coumarin, 396 nm; and MIANS, 325 nm.

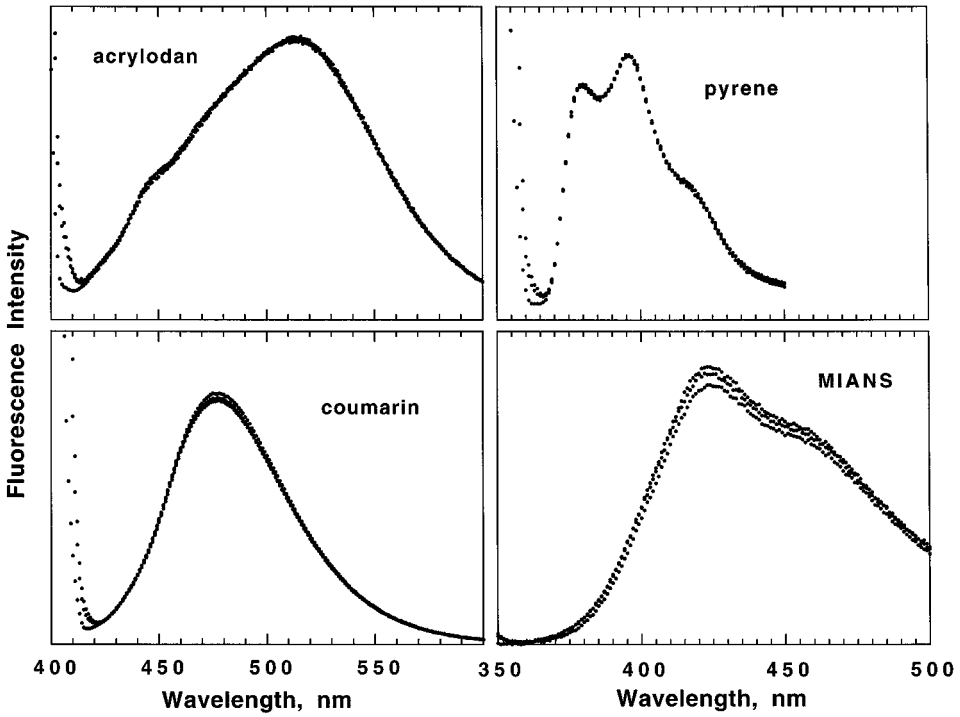

FIGURE 2: Fluorescence spectra of Cys-153-labeled CaD with or without actin (A) and with or without tropomyosin (Tm). Since there is very little, if any, change in CaD fluorescence upon adding actin (with or without Tm), the separate curves are not labeled. For acrylodan, actin = smooth and CaD = heat-treated. For pyrene, actin = smooth and CaD = native. For coumarin, actin = smooth and CaD = native. For MIANS, actin = skeletal and CaD = heat-treated. λ_{ex} values of probes are as in Figure 1.

and polarization of a rhodamine probe attached to Cys-580 in the COOH domain of caldesmon was very sensitive to the binding of actin (with or without Tm), whereas the probe at Cys-153 in the NH_2 domain was not responsive (Graceffa, 1995). In this study, we have extended such experiments to include a variety of additional fluorescence probes in order to rule out the possibility that the results for the rhodamine probe at Cys-153 were an artifact of that particular probe. For all probes, the results were the same as those for the rhodamine probe. The fluorescence intensity of the acrylodan, pyrene, coumarin, and MIANS probes at Cys-580 increased dramatically upon binding to actin with smaller further increases in the presence of tropomyosin (Figure 1). For the acrylodan probe, there was, in addition, a large blue shift in the peak fluorescence indicating a more hydrophobic environment for the probe (Prendergast et al., 1983) upon

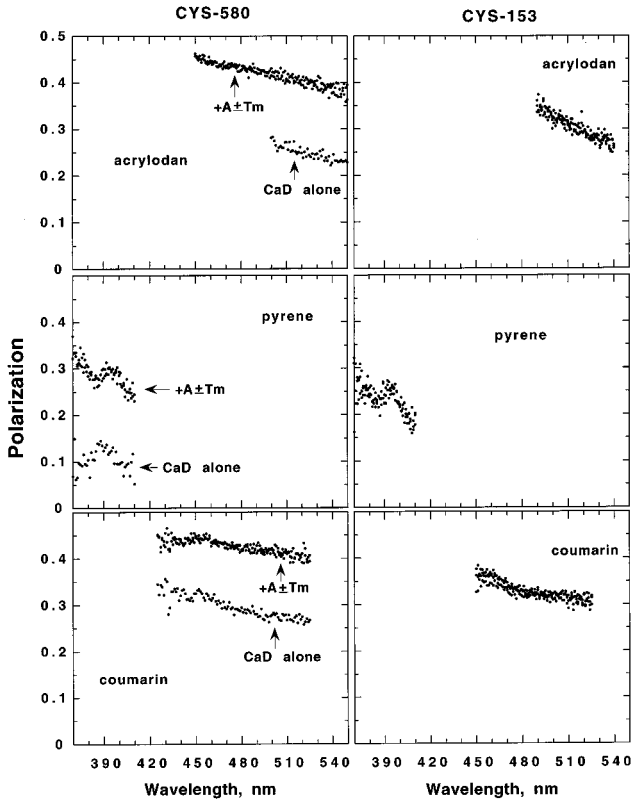

FIGURE 3: Fluorescence polarization spectra of Cys-580- and Cys-153-labeled CaD with or without actin (A) and with or without tropomyosin (Tm). For Cys-580-labeled, CaD = heat-treated; for Cys-153-labeled, CaD = heat-treated for acrylodan and native for pyrene and coumarin. Actin = skeletal. For Cys-153-labeled CaD, the curves are not labeled since they all overlap. λ_{ex} values are as in Figure 1.

binding to actin. The coumarin probe showed a similar, but smaller, blue shift. In the presence of gizzard smooth muscle actin, these changes were much greater for the acrylodan probe (Figure 1) and only slightly greater for the other probes (not shown). The polarization of probes at Cys-580 also exhibited large increases upon binding to actin (with or without Tm), indicating decreased mobility of the probes for actin-bound caldesmon (Figure 3). For the same probes attached to Cys-153, in the NH_2 domain of caldesmon, there were essentially no changes in fluorescence intensity, peak maximum, or polarization upon binding to actin (with or without Tm) regardless of whether rabbit skeletal or gizzard smooth muscle actin and whether heat-treated or native caldesmon were used (Figures 2 and 3).

Fluorescence Resonance Energy Transfer. The above results are consistent with the idea that while the COOH domain of caldesmon is bound to actin the NH_2 domain is, for the most part, dissociated from actin. If this is the case, then the distance between the NH_2 domain and actin should be greater than that between the COOH domain and actin. Therefore, the distance between Cys-374 of actin and Cys-580 or Cys-153 of caldesmon was estimated from fluorescence resonance energy transfer from a fluorescence donor, specifically attached to each of the caldesmon cysteines, to a fluorescence acceptor specifically attached to Cys-374 of actin. With a coumarin−maleimide (CPM) probe on Cys-580 and a fluorescein−maleimide (FM) probe on Cys-374 of actin, there was a 76% decrease in CPM steady-state fluorescence (Figure 4A), i.e. a 76% energy transfer efficiency (E), calculated as described in Experimental Procedures. The transfer effeciency was the same with the additional presence of tropomyosin (data not shown). From an R_o of 48 Å for this donor/acceptor pair, one calculates (see Experimental Procedures) a distance of 39 Å between the probes in the caldesmon−actin protein complex. Distances from fluorescence resonance energy transfer are most accurate when the transfer effeciency is close to 50%. Therefore, we measured fluorescence resonance energy transfer between probes on caldesmon Cys-580 and actin Cys-374 in the presence of tropomyosin using the IAEDANS/DDPM donor/acceptor pair with a lower R_o of 27 Å. For this pair, the transfer efficiency from steady-state fluorescence was 52% (Figure 5) and the resulting calculated distance was 26.5 Å. The transfer efficiency of the IAEDANS/DDPM pair was also determined by fluorescence lifetime measurements. The fluorescence decay of IAEDANS attached to caldesmon Cys-580 changed from a single lifetime of 14.0 ns for caldesmon alone (data not shown), to

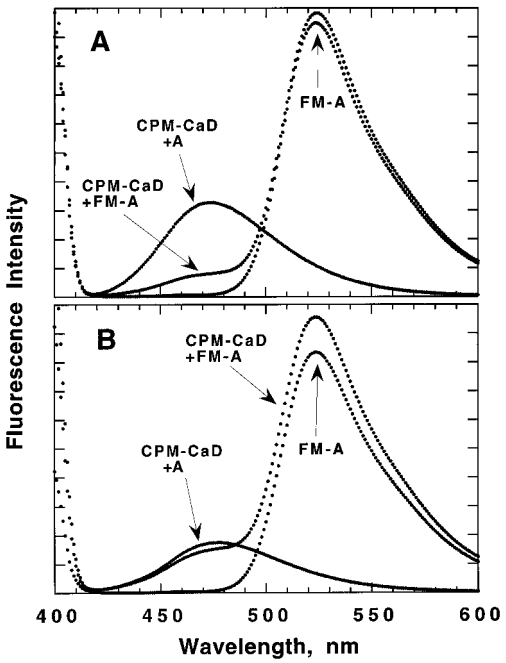

FIGURE 4: Steady-state fluorescence resonance energy transfer between the FM acceptor on Cys-374 of actin (FM-A) and the CPM donor on Cys-580 (A) or Cys-153 (B) of caldesmon (CPM−CaD). The fluorescence spectrum of FM−actin alone, at the excitation wavelength of CPM, is shown because of its significant contribution in the region of the CPM spectrum. A = actin. λ_{ex} of CPM = 396 nm.

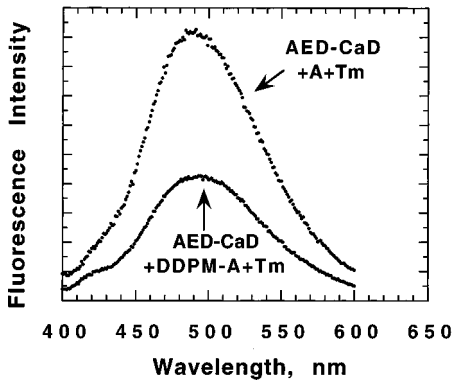

FIGURE 5: Steady-state fluorescence resonance energy transfer from IAEDANS on caldesmon Cys-580 (AED−CaD) to DDPM on actin Cys-374 (DPPM−A), in the presence of tropomyosin (Tm). A = actin. λ_{ex} of IAEDANS = 340 nm.

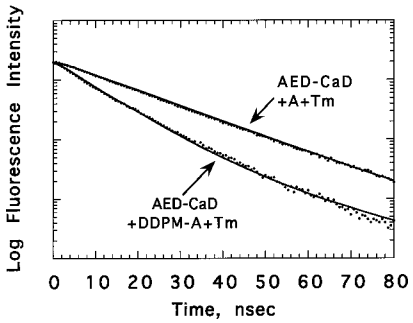

FIGURE 6: Lifetime fluorescence resonance energy transfer from IAEDANS on caldesmon Cys-580 (AED−CaD) to DDPM on actin Cys-374 (DPPM−A) in the presence of tropomyosin (Tm). A = actin. The experimental fluorescence decay is shown by closed circles, and solid lines are calculated curves from the method-of-moment analysis (see Experimental Procedures). The decay in the presence of unlabeled actin was adequately described by a single exponential with a lifetime of 16.0 ns. In the presence of labeled actin, the decay was adequately described by a major component (93% contribution) with an 8.5 ns lifetime and a minor component (7% contribution) with a 20 ns lifetime.

a single lifetime of 16.0 ns in the presence of unlabeled actin (with Tm), to a single major component (93% contribution) with a lifetime of 8.5 ns in the presence of DDPM-labeled actin (with Tm) (Figure 6), corresponding to a transfer effeciency of 46.5%. This translates to an interprobe distance of 27.5 Å, in agreement with the steady-state determination. This distance of about 27 Å between labels on actin Cys-374 and caldesmon Cys-580 is probably more accurate than the 39 Å determined for the CPM/FM pair (see the discussion of κ^2, below). From sedimentation assays, it was determined that close to 100% of the labeled caldesmons bound to the labeled actins, indicating that it was unnecessary to make any corrections to the calculated distances due to free caldesmon.

For the CPM probe at Cys-153 of caldesmon, the energy transfer efficiency to FM−actin was small (Figure 4B), ranging from 5 to 17% for different samples, corresponding to a distance of from 65 to 80 Å between probes. The energy transfer efficiency was not significantly affected by the additional presence of tropomyosin or by the substitution of Cys-153-labeled native caldesmon for Cys-153-labeled heat-treated caldesmon (data not shown). (For native caldesmon, the transfer efficiency was less than 10%.) Close to 100% of the CPM-labeled caldesmon bound to the FM-labeled actin, indicating that no further correction to the distance calculations was necessary. The absolute value of this distance is rather uncertain because of the low level of energy transfer. In order to estimate a more accurate distance between caldesmon Cys-153 and actin, it would be necessary to use a donor/acceptor pair with a much larger R_o. However, such an appropriate pair is not readily available (Wu & Brand, 1994). In spite of this limitation, it is clear that the distance between caldesmon Cys-153, in the NH_2 domain, and actin Cys-374 is much greater than that between caldesmon Cys-580, in the COOH domain, and actin Cys-374. Thus, fluorescence resonance energy transfer experiments also support the idea of a dissociated NH_2 domain of actin-bound caldesmon.

The distances determined by fluorescence resonance energy transfer depend on the assumption that the orientation factor κ^2 has a value of $^2/_3$. The validity of this assumption is difficult to assess and has been the subject of much discussion [Stryer (1978), Wu and Brand (1994), and Selvin (1995) for reviews]. κ^2 is $^2/_3$ if both donor and acceptor are free to rotate without any restricted orientation. If both donor and acceptor are fixed in one particular orientation, then κ^2 can vary anywhere from 0 to 4, depenending on the orientation. However, in reality, κ^2 can be taken to be $^2/_3$ with an uncertainty in R_o often less than 10% and usually less than 20% if (1) the donor has multiple electronic transitions with mixed polarizations (Haas et al., 1978), (2) the donor or acceptor is fixed but has some random orientation, or (3) the donor and/or acceptor are not rigidly fixed but have some mobility (Stryer, 1978; Wu & Brand, 1994; Selvin, 1995). For the two donor/acceptor pairs which we have used, either the donor or acceptor appears to have considerable mobility since the value of their fluorescence polarization is considerably less than the limit found for the rigid probe. For the CPM/FM pair, CPM on CaD−Cys-580 appears to be almost completely immobile and without mixed polarizations in the presence of actin/Tm since its polarization is about 0.44 (Figure 3), close to the theoretical maximum of 0.5, whereas CPM on CaD−Cys-153 has some mobility since its polarization is about 0.33 in the presence of actin/Tm (Figure 3). However, FM on actin, in the presence of Tm and CaD, appears to have a high degree of mobility since it has a low polarization value of 0.16 (data not shown) compared to the experimental rigid limit of 0.43 (this work; Chen & Bowman, 1965). The IAEDANS label on CaD, in the presence of actin and Tm, also has a low polarization of 0.17 (data not shown) compared to its experimental rigid limit of about 0.35 (Hudson & Weber, 1973), indicating considerable mobility of that probe. Furthermore, a low experimental rigid limit polarization of the IAEDANS donor (0.35), compared to the theoretical limit of 0.5, suggests multiple transitions with mixed polarizations, which also reduces the uncertainty in using a κ^2 value of $^2/_3$ (Haas et al., 1978). It is not possible to asses the mobility of the DDPM acceptor since it is nonfluorescent. However, any mobility of DDPM will further decrease the uncertainty in the use of $\kappa^2 = {}^2/_3$ for the IAEDANS/DDPM pair. Therefore, with the possible exception of the CPM(Cys-580)-CaD/FM(Cys-374)actin pair, the error in distances due to the assumption of $\kappa^2 = {}^2/_3$ should be small compared to the large difference in distances that we have estimated between the end domains of caldesmon and actin. The low mobility of CPM on CaD Cys-580, in the presence of actin/Tm, may result in greater uncertainty and error due to the $\kappa^2 = {}^2/_3$ assumption and might account for the difference in distance measured with this pair (39 Å) compared to that with the IAEDANS/DDPM pair (27 Å).

Fluorescence Quenching. The possible interaction of the two ends of caldesmon with actin was also monitored by the acrylamide quenching of fluorescence of probes at Cys-580 and Cys-153 of caldesmon in the presence and absence of actin (with or without Tm). Quenching of the fluorescence of the acrylodan probe at both Cys-580 and Cys-153 was reduced in the presence of actin and somewhat further reduced in the additional presence of tropomyosin (Figure 7), indicating a shielding of the probe from solvent by the binding of actin (with or without Tm). The reduction in quenching by actin was much greater for acrylodan at Cys-580 than for the probe at Cys-153. The same qualitative effects were observed for the pyrene probe (data not shown). In order to quantitate the probe accessibility to quencher,

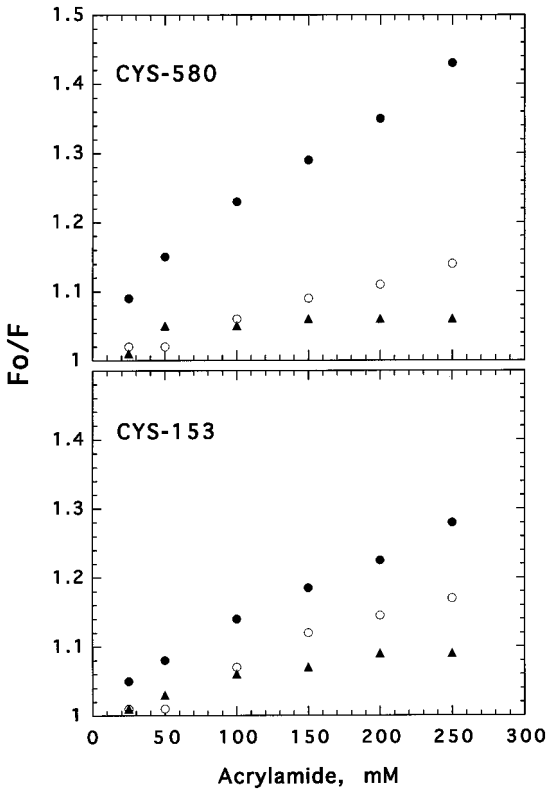

FIGURE 7: Fluorescence quenching by acrylamide of the acrylodan probe attached to caldesmon at Cys-580 or Cys-153: caldesmon alone (closed circles), caldesmon and actin (open circles), and caldesmon, actin, and Tm (closed triangles).

Table 1: Relative Accessibility to Acrylamide Quencher of Acrylodan Probe Attached to Caldesmon (CaD) Cysteines, in the Presence/Absence of Actin

CaD cysteine-labeled	actin[a]	$k_q/(k_q$ for CaD alone with probe at Cys-580)
580	−	1.0
580	+	0.21
153	−	0.585
153	+	0.41

[a] +, with; −, without.

we calculated the bimolecular quenching constant (k_q), relative to the acrylodan probe attached to Cys-580 for caldesmon alone, as detailed in Experimental Procedures. The results of this determination (Table 1) are that, first of all, in the absence of actin, acrylodan at Cys-153 of caldesmon is more shielded from quencher than the probe at Cys-580, being quenched at about 60% of the rate of the probe at Cys-580. This indicates that, in the absence of actin, acrylodan−Cys-580 is more exposed to solvent than acrylodan−Cys-153. However, in the presence of actin, acrylodan−Cys-580 becomes much less exposed to quencher than acrylodan−Cys-153, since in the presence of actin the quenching rate of acrylodan at Cys-580 is 50% of that of the probe at Cys-153 (Table 1). These quenching results suggest that the Cys-580 region of the COOH domain of caldesmon is in closer proximity with actin than is the Cys-153 region of the NH_2 domain, but that the Cys-153 region is in the general vicinity of actin or spends some time close to the actin filament.

Native Thin Filaments. The above experiments were performed with reconstituted thin filaments. These same kinds of experiments cannot be performed with native thin filaments which preclude the isolation and labeling of individual thin filament proteins. However, it was thought that labeled caldesmon added to gizzard native thin filaments might bind to actin since these native thin filament preparations, which contain predominantly actin, tropomyosin, and caldesmon, contain some caldesmon-free actin filaments (Lehman et al., 1989). Indeed, when caldesmon labeled at Cys-580 with acrylodan was added to native thin filaments, at an actin/labeled-caldesmon molar ratio of roughly 40, the acrylodan fluorescence intensity was enhanced and the fluorescence peak was blue shifted (Figure 8), as when added to reconsituted thin filaments (Figure 1). Also as with reconstituted thin filaments, the fluorescence intensity and peak position of Cys-153 acrylodan-labeled caldesmon remained almost completely unchanged upon addition to native thin filaments (Figure 8). The polarization (in the 515−530 nm wavelength range) of the Cys-153-labeled protein in the presence of native thin filaments was 0.28 ± 0.01, about the same as that for the free caldesmon (Figure 3). These results indicate that the added caldesmon is binding to native actin filaments and suggests that, whereas the caldesmon COOH domain is actin-bound, the NH_2 domain is mostly dissociated.

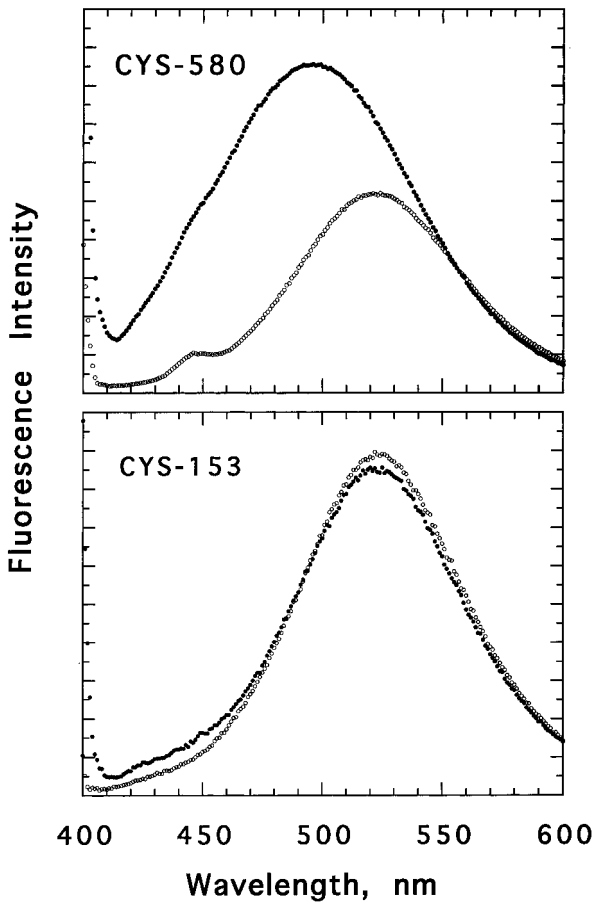

FIGURE 8: Fluorescence spectra of acylodan-labeled Cys-580 or Cys-153 of caldesmon, free (open circles) and bound (closed circles) to native thin filaments. λ_{ex} of acrylodan = 385 nm.

DISCUSSION

We have investigated the interaction of the COOH and NH_2 domains of intact caldesmon with actin filaments in solution, by monitoring several fluorescence properties of a variety of probes specifically attached to Cys-580 in the

COOH domain or Cys-153 in the NH_2 domain. For probes at Cys-580, the fluorescence intensity, peak position, polarization, and acrylamide quenching changed dramatically upon the addition of actin (with or without Tm), indicating both shielding of the probe from solvent and immobilization of the probe upon binding to actin. For probes attached to Cys-153, there were no or very little changes in these fluorescence properties upon binding of caldesmon to actin (with or without Tm), indicating that probes at Cys-153 hardly sense the presence of actin. From fluorescence resonance energy transfer experiments, we estimated a distance of 27 Å between caldesmon Cys-580 and actin Cys-374 and 65−80 Å between caldesmon Cys-153 and actin Cys-374. These distance measurements coupled with the above fluorescence results strongly suggest that, whereas the COOH domain of caldesmon is bound to actin, the NH_2 domain of caldesmon is mainly dissociated. This conclusion is supported by reports that COOH-terminal fragments of caldesmon bind to actin whereas NH_2-terminal fragments do not (Szpacenko & Dabrowska, 1986; Fujii et al., 1987; Katayama et al., 1989; Hayashi et al., 1991; Redwood & Marston, 1993) and that caldesmon Cys-580 can be cross-linked to actin with high yield whereas Cys-153 can be cross-linked with very low yield, at most (Graceffa & Jancso, 1991; Graceffa et al., 1993; Graceffa, 1995). The degree of the NH_2-terminal part of caldesmon which is dissociated from actin must extend beyond Cys-153 to explain our results and could possibly include the central domain since neither NH_2-terminal fragments of caldesmon which contain this domain (Szpacenko & Dabrowska, 1986; Fujii et al., 1987; Katayama et al., 1989; Hayashi et al., 1991; Redwood & Marston, 1993) nor central domain fragments alone (Wang et al., 1991; Redwood & Marston, 1993) bind to actin.

We cannot entirely rule out the possibility that the NH_2 domain is bound to actin and that probes at caldesmon Cys-153 point away from the actin-binding site such that their cross-linking and fluorescent properties are insensitive to actin binding and that there is a large distance between such a probe and a probe on actin Cys-374. However, since caldesmon is an extended and thin molecule (Furst et al., 1986; Lynch et al., 1987; Graceffa et al., 1988; Mabuchi & Wang, 1991), except for globular structure in the COOH domain (Levine et al., 1990; Graceffa & Jancso, 1993; Huber et al., 1996), it would be expected that NH_2 domain binding to actin would affect the environment or mobility of probes at Cys-153, at least indirectly by some protein conformational change. The polarization of probes at Cys-153 has "intermediate" values (Figure 3), suggesting that they are somewhat immobilized on caldesmon and thus should reflect changes in mobility of the region to which they are attached. Furthermore, if the NH_2 domain of caldesmon is bound to actin, it appears improbable that even a probe oriented away from actin could be measured to be 65−80 Å from actin Cys-374 by FRET. This is the case, since an inspection of a three-dimensional model of the actin filament (Holmes et al., 1990) demonstrates that, on the actin surface, it is not possible to get any farther away from an actin Cys-374 than about 37 Å, whereupon one approaches even closer to a Cys-374 of another actin subunit in the filament. Even at a maximum distance from actin Cys-374 [∼37 Å + ∼15 Å (width of caldemson) + ∼10 Å (length of the CPM donor probe)], the donor probe on caldesmon would then be approximately equidistant from several FM acceptors which would all contribute to the energy transfer efficiency and yield an apparent distance much less than the R_o (49 Å) of the CPM/FM pair [see Richter et al. (1985)].

Most studies of the interaction between caldesmon and actin use caldesmon prepared with a heat treatment step and actin prepared from rabbit skeletal muscle, because of the ease and convenience of these preparations. In this work, we also conducted our studies with native caldesmon, i.e. prepared without a heat treatment step, since heat might irreversibly alter the properties of caldesmon, and with smooth muscle actin with which caldesmon is associated in vivo. In particular, a recent microscopy study showed that caldesmon prepared without heat exhibits more lengthwise binding to actin than heat-treated caldemson (Zhuang et al., 1996). However, in our work, the substitution of native caldesmon for heat-treated caldesmon and/or smooth muscle actin for skeletal muscle actin had no significant effect on our fluorescence (this study) or photo-cross-linking (Graceffa, 1995) results. This leads us to conclude that, although native caldesmon might bind lengthwise to actin more so than heat-treated caldesmon, the NH_2 domain of native or heat-treated caldesmon is mostly dissociated from (skeletal or smooth muscle) actin.

It has also been demonstrated, in this work, that actin shields fluorescence probes at caldesmon Cys-153 from an acrylamide quencher, albeit much less than for probes at Cys-580, and, in a previous study (Graceffa, 1995), that Cys-153 can be disulfide cross-linked to Cys-374 of actin, albeit much less and much slower than Cys-580. These findings suggest that, although the NH_2 domain of actin-bound caldemson is, for the most part, dissociated from actin, it spends some time close to the actin filament possibly by a weak interaction with actin, and/or by collisions with actin, and/or because of restricted flexibility which constrains the NH_2 domain to be in the vicinity of actin. In the last possibility, the NH_2 domain would not fully project away from the actin filament, which might account for the results of the microscopy studies that do not observe caldesmon projections (see below).

Native thin filaments, consisting of actin, tropomyosin, and caldesmon, cannot be studied by the techniques we have used with reconstituted thin filaments since it is not possible to specifically label caldesmon in this protein complex. However, we did add acrylodan-labeled caldesmon to gizzard native thin filaments and found, as with reconstituted thin filaments, that the fluorescence properties of the Cys-580-labeled protein were sensitive to binding whereas those of the Cys-153-labeled protein were not. These results suggest that the NH_2 domain of caldesmon is also dissociated in native thin filaments. However, we cannot rule out the possibility that the added caldesmon might bind to native actin filaments differently than caldesmon assembled in vivo or might bind to cytoskeletal, and not contractile, native actin filaments (Furst et al., 1986; Lehman et al., 1987), both of which are present in this native thin filament preparation (Lehman et al., 1989), but only the latter containing caldesmon (Furst et al., 1986; North et al., 1994; Mabuchi et al., 1996). In any case, the added caldesmon binds to actin filaments assembled in vivo in a similar manner as it does to extracted actin reassembled in vitro.

The arrangement of intact caldesmon on actin filaments has also been studied by electron microscopy of fixed samples (Moody et al., 1990; Mabuchi et al., 1993; Katayama

& Ikebe, 1995) and X-ray microscopy of highly concentrated thin filament sols (Popp & Holmes, 1992). These studies are controversial in that two of the studies (Moody et al., 1990; Popp & Holmes, 1992) observe no or occasional caldesmon projections in either reconstituted or native thin filaments, one study (Mabuchi et al., 1993) observes caldesmon projections in reconsituted but not native thin filaments, and one study (Katayama & Ikebe, 1995) observes projections in both thin filaments. It is unclear what underlies these differences. Possibly, the different sample preparations, treatments, and conditions might affect the state of the caldesmon. Another possibility is that the NH_2 domain can exist in an actin filament-associated and an actin filament-dissociated state and that the equilibrium or switching between these states is controlled by conditions or factors which have yet to be discovered. For example, although the caldesmon NH_2 domain binds to tropomyosin in the absence of actin, it does not bind to tropomyosin in the presence of actin (Tsuruda et al., 1995; Graceffa, 1995), which is consistent with the lack of an effect of tropomyosin on the fluorescence of the NH_2 domain in the present work. It could be possible that under certain conditions the caldesmon NH_2 domain binding site on actin-bound tropomyosin might be exposed to the NH_2 domain which would thereby be anchored to the actin filament. Those studies which do not observe projections conclude that the NH_2 domain is interacting with actin. However, it might be possible that, although projections of caldesmon cannot be observed, the NH_2 domain is dissociated but not fully projecting, as our findings suggest (see above), or that the part of caldesmon which is dissociated is relatively small and thus, in either case, not observable by microscopy.

In summary, our results strongly suggest that a major part of the NH_2 domain of actin-bound caldesmon is, for the most part, dissociated from reconstituted and native actin filaments. However, we cannot decide on the extent of the N-terminal part of caldesmon which is dissociated and how far the dissociated part extends away from the actin filament, the details with which we might be able to resolve some of the differences with microscopy studies. Since the NH_2 domain of caldesmon binds to myosin in its neck region (Ikebe & Reardon, 1988; Hemric & Chalovich, 1990) and the COOH domain binds to actin, and since it has been shown that caldesmon can cross-link actin and myosin filaments (Haeberle et al., 1992; Katayama et al., 1995; Marston et al., 1992; Hemric et al., 1994), a dissociated NH_2 domain might be the molecular basis for this cross-linking ability. The NH_2 domain might have to be dissociated in order to interact with myosin's neck (i.e. S2) region which does not come in contact with actin.

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