Caldesmon, N-Terminal Yeast Actin Mutants, and the Regulation of Actomyosin Interactions[†]

Rachelle H. Crosbie,[‡] Carl Miller,[‡] Joseph M. Chalovich,[§] Peter A. Rubenstein,[‖] and Emil Reisler*,[‡]

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024, Department of Biochemistry, East Carolina University School of Medicine, Greenville, North Carolina 27858, and Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

Received August 2, 1993; Revised Manuscript Received January 7, 1994[®]

ABSTRACT: N-Terminal yeast actin mutants were used to assess the role of N-terminal acidic residues in the interactions of caldesmon with actin. The yeast actins differed only in their N-terminal charge: wild type, two negative charges; 4Ac, four negative charges; DNEQ, neutral charge; ΔDSE, one positive charge. Caldesmon inhibition of actomyosin subfragment 1 ATPase was affected by alterations in the N-terminus of actin. This inhibition was similar for skeletal muscle α-actin and the yeast 4Ac and wild-type actins (80%), but much smaller for the neutral and deletion mutants (15%). However, cosedimentation experiments revealed similar binding of caldesmon to polymerized rabbit skeletal muscle α-actin and each yeast actin. This result shows that the N-terminal acidic residues of actin are not required for the binding of caldesmon to F-actin. Caldesmon–actin interactions were also examined by monitoring the polymerization of G-actin induced by caldesmon. Although the final extent of polymerization was similar for all actins tested, the rates of polymerization differed. Skeletal muscle and 4Ac actins had similar rates of polymerization, and the wild-type actin polymerized at a slower rate. The neutral and deletion mutants had even slower rates of polymerization by caldesmon. The slow polymerization of DNEQ G-actin was traced to a greatly reduced binding of caldesmon to this mutant G-actin when compared to wild-type and α-actin. $MgCl_2$-induced actin polymerization proceeded at identical rates for all actins. These results, which are discussed in terms of multiple contact sites between actin and myosin subfragment 1 and caldesmon, contrast the regulatory with the binding interactions of caldesmon with the N-terminus of actin.

Regulation of the interaction of actin and myosin by caldesmon (CDS[1]), in response to calmodulin, has been postulated as one of the two major regulatory mechanisms of smooth muscle contractility (Chalovich, 1993; Marston & Redwood, 1991; Sobue & Sellers, 1991). *In vitro*, caldesmon inhibits the superprecipitation of actomyosin (Sobue *et al.*, 1982) and the actin-activated ATPase activity of myosin and its subfragments (Marston *et al.*, 1984; Ngai & Walsh, 1984; Lash *et al.*, 1986; Chalovich *et al.*, 1987; Lim & Walsh, 1988). Recent studies with caldesmon fragments and smooth muscle fibers (Pfitzer *et al.*, 1993; Katsuyama *et al.*, 1992) demonstrate the physiological feasibility of the regulation of smooth muscle contraction by caldesmon.

A first step in understanding the molecular basis of this control mechanism is to determine how caldesmon interacts with actin and myosin. Several studies indicate that caldesmon inhibits the binding of myosin–ATP to actin, irrespective of the source of myosin, possibly by competing with myosin for overlapping binding sites on actin[2] (Chalovich *et al.*, 1987; Hemric *et al.*, 1988; Velaz *et al.*, 1989; Horiuchi *et al.*, 1989; Brenner *et al.*, 1991; Nowak *et al.*, 1989; Bartegii *et al.*, 1990; Haeberle *et al.*, 1992). One proposed area of overlap between caldesmon and S-1–ATP is the N-terminus of actin, particularly the first seven residues. Antibodies directed against these residues of actin inhibit the binding of S-1–ATP to actin and have a lesser effect on rigor S-1 binding (Sutoh, 1982; DasGupta & Reisler, 1989, 1992; Bertrand *et al.*, 1989). The same antibodies partially inhibit the binding of intact caldesmon to actin and, to a lesser extent, that of the 20-kDa fragment of caldesmon (Adams *et al.*, 1990). Interestingly, total inhibition was not achieved, suggesting the presence of other binding sites. Chemical cross-linking of S-1 (Sutoh, 1982) and caldesmon (Bartegi *et al.*, 1990) to actin, as well as NMR spectroscopy of the S-1–actin (Trayer *et al.*, 1987) and caldesmon–actin (Patchell *et al.*, 1989) complexes, also suggests that the binding of both proteins involves the N-terminal region of actin. However, the above approaches suffer from possible steric effects of antibodies or effects of nonspecific cross-linking, and they do not test the functional significance of the postulated binding of caldesmon to the N-terminus of actin.

A method to map sites of protein contact while avoiding some ambiguities of interpretation involves the use of site-directed mutagenesis. Amino-terminal deletion mutants of actin have been generated in *Saccharomyces cerevisiae* (Cook *et al.*, 1992, 1993), *Dictyostelium* (Sutoh *et al.*, 1991), and chicken β-actin (Aspenstrom & Karlsson, 1991) to monitor the interaction of myosin with actin. Mutations in the N-terminal region of actin resulted in alterations in actomyosin

---

[†] This work was supported by grants from the United States National Institutes of Health and National Science Foundation to E.R. (AR22031 and MCB9206739, respectively), J.M.C. (NIH AR35216 and AR40540-01A1), and P.A.R. (NIH GM33689) and by USPHS National Research Service Award GM to C.M. (07185).

[‡] University of California at Los Angeles.

[§] East Carolina University School of Medicine.

[‖] University of Iowa.

[®] Abstract published in *Advance ACS Abstracts*, February 15, 1994.

[1] Abbreviations: CDS, caldesmon; CPM, 7-(diethylamino)-3-(4′-maleimidophenyl)-4-methylcoumarin; F-actin, filamentous (polymerized) actin; G-actin, monomeric actin; S-1, myosin subfragment 1.

[2] The importance of this competition in the inhibition of ATPase activity is not universally accepted [for example, see Marston and Redwood (1992)]. However, there is general agreement that this competition is responsible for the inhibition of ATPase activity in the absence of tropomyosin.

0006-2960/94/0433-3210$04.50/0

ATPase and motility, but did not decrease the binding of S-1 to actin. *In this work, we show that mutations in the N-terminus of actin have little effect on caldesmon binding to F-actin, but a large effect on the ability of caldesmon to inhibit acto-S-1 ATPase and to bind monomeric actin.* In contrast to previous observations, these results show that the N-terminal acidic residues of actin do not significantly contribute to caldesmon binding to filamentous actin. However, these residues are essential for the inhibition of acto-S-1 ATPase activity and are important for the binding and polymerization of G-actin by caldesmon.

MATERIALS AND METHODS

Reagents. ATP, Folin and Ciocalteu's phenol reagent, and β-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Yeast extract, dextrose, and Bacto-peptone were purchased from Difco (Detroit, MI). DNaseI and the Bradford protein assay reagents were purchased from Worthington Enzymes (Freehold, NJ) and Bio-Rad (Richmond, CA), respectively. *N*-(1-Pyrenyl)iodoacetamide and 7-(diethylamino)-3-(4′-maleimidophenyl)-4-methylcoumarin (CPM) were obtained from Molecular Probes (Junction City, OR). Distilled and Millipore-filtered water and analytical grade reagents were used in all experiments.

Yeast Strains and Mutagenesis. Construction of the yeast actin mutants was accomplished according to an established protocol (Cook *et al.*, 1992, 1993). The haploid strains TDyDD, TDyDNEQ, and KC26 have been described previously (Cook *et al.*, 1992).

Preparation of Proteins. The yeast actins used in this study were purified by DNaseI affinity chromatography as previously described (Cook *et al.*, 1992). Rabbit skeletal muscle α-actin was prepared in G-actin buffer (5 mM Tris, 0.5 mM β-mercaptoethanol, 0.2 mM ATP, and 0.2 mM $CaCl_2$ (pH 7.6)) by the procedure of Spudich and Watt (1971). Myosin subfragment 1 (S-1) was purified as described previously (Godfrey & Harrington, 1970; Weeds & Pope, 1977). Caldesmon was purified from turkey gizzards as described by Velaz *et al.* (1989). Caldesmon was concentrated by ultrafiltration on a YM-2 membrane (Amicon), frozen in dry ice/acetone, and shipped to UCLA in a buffer containing 100 mM NaCl, 10 mM imidazole (pH 7.4), and 1 mM DTT. Caldesmon was incubated with 1 mM DTT prior to use in experiments. Caldesmon concentrations were determined by the Lowry assay (1951) using BSA as a standard. The molecular weight of caldesmon (87 000) used in this work is the value assigned by cDNA sequencing of this protein (Bryan *et al.*, 1989).

Pyrene Modification of α-Actin. Rabbit skeletal muscle α-actin was labeled at Cys-374 with *N*-(1-pyrenyl)iodoacetamide following the method established by Cooper *et al.* (1983). The extent of labeling was measured by using a molar extinction coefficient of $E_{344} = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ for the protein–dye complex (Kouyama & Mikashi, 1981). The labeling stoichiometry ranged between 0.80 and 1.00 probe/actin. The protein concentrations of modified actin were measured using the Bradford assay (1976).

Airfuge Binding Experiments. Rabbit skeletal muscle (4 μM) and yeast (4 μM) actins were polymerized with 2 mM $MgCl_2$ and subsequently incubated with caldesmon (0.25–3.5 μM), resulting in a range of caldesmon to actin ratios of 0.06–0.90. The protein mixtures were centrifuged at 160000*g* for 20 min in an air-driven ultracentrifuge (Beckman Instruments). Under these conditions, caldesmon was not pelleted when centrifuged in the absence of actin. The pelleted samples were resolubilized with G-buffer. Supernatant and resolubilized pellet fractions were denatured and analyzed on either 10 or 15% polyacrylamide gels (Laemmli, 1970). Coomassie Blue R-stained protein bands were scanned with a Biomed Instruments (Fullerton, CA) scanning densitometer interfaced to a DTK computer. The densitometric traces of the scanned protein bands were analyzed to determine the molar ratios of caldesmon bound to actin. Molar ratios of bound proteins were calculated using molar stain ratios obtained from the appropriate calibration gels.

Fluorescence and Light-Scattering Measurements for Polymerization. Fluorescence intensities of pyrene-labeled actin were measured in the Spex Fluorolog spectrophotometer (Spex Industries, Inc., Edison, NJ) as previously described (Miller *et al.*, 1988). For pyrene fluorescence measurements, the excitation and emission monochromators were set at 368 and 407 nm, respectively. Light-scattering measurements were carried out at wavelengths of 325 nm for both excitation and emission. When the polymerization of actin was followed by pyrene fluorescence measurements, 10% of the total actin was pyrenyl-α-actin. Pyrene actin did not interfere with the polymerization of yeast actins since the labeled actin was not polymerized by caldesmon or $MgCl_2$ at these low actin concentrations. Pyrene modification of the yeast actins was not attempted because of their limited quantities in each preparation.

Acto-S-1 ATPase Activity. Acto-S-1 ATPase activity was measured using the malachite green assay of Kodama *et al.* (1986). In brief, rabbit, wild-type, or 4Ac actin (4 μM) was polymerized with 2 mM $MgCl_2$ in a buffer consisting of 10 mM KCl and 10 mM imidazole (pH 7.0). The 100-μL samples were subsequently incubated at 25 °C with 0.4 μM S-1 and a range of caldesmon concentrations to give caldesmon to actin ratios of 0.013–0.15. The ATPase reaction was initiated by the addition of 3 mM ATP. The low-salt conditions of the ATPase assay were selected in order to enhance acto-S-1 binding in the presence of ATP. The reactions were stopped with 0.6 M perchloric acid and then diluted to a final concentration of 0.3 M perchloric acid. The mixture was centrifuged at 14 000 rpm for 10 min in a microcentrifuge (Eppendorf) to remove the precipitated proteins. Equal volumes of the supernatant and the malachite green reagent (Kodama *et al.*, 1986) were mixed. The samples were incubated at 25 °C for 20 min to allow for color development. The color intensity was measured at 650 nm (Hewlett-Packard diode array spectrophotometer). Acto-S-1 ATPase activities for DNEQ were measured as above, except at actin concentrations of 30, 14, and 7 μM. In these cases, actin filaments were pelleted to remove inorganic phosphate and resuspended in the polymerization buffer prior to mixing with S-1. S-1 ATPase activities were also measured for ΔDSE actin at a concentration of 7 μM actin. Higher concentrations of this actin mutant were not used due to its tendency to bundle (Cook *et al.*, 1992). All acto-S-1 ATPase activities were corrected for the ATPase of S-1 alone.

Fluorescence Measurements of Caldesmon Binding to Labeled G-Actin. Rabbit α-, wild-type, and DNEQ actin (3 μM) in 5 mM Tris, 0.2 mM $CaCl_2$, and 0.2 mM ATP (pH 7.6) were labeled with the coumarin probe, CPM (3 μM). The labeling reaction was monitored by an increase in coumarin fluorescence (excitation and emission wavelengths were 385 and 470 nm, respectively). The labeling was stopped with 1 mM DTT when the fluorescence reached plateau values, indicating completion of the reaction (the plateaus were reached in approximately 5 min). A comparison of the emission spectra of CPM-labeled G-actin (excitation wavelength, 385 nm) revealed a caldesmon-induced blue shift in the fluorescence of coumarin actin. This spectral difference between free and bound G-actin was used in subsequent measurements to assess the binding of caldesmon to monomeric actin. Thus, the binding of caldesmon to CPM-labeled G-actin was determined *via* fluorescence emission changes at 435 nm (excitation wavelength, 385 nm). Fluorescence measurements were recorded immediately after caldesmon addition to insure that the reported values reflected the binding to monomeric actin.

RESULTS

The involvement of the N-terminal region of actin in the binding of caldesmon was tested directly by using yeast actin mutants. The four yeast actins employed in this work differed only at the N-terminus:

4Ac actin Ac-Met-Asp-Glu-Asp-Glu-Val
(4 negative charges)

wild-type actin Ac-Met-Asp-Ser-Glu-Val
(2 negative charges)

DNEQ actin Ac-Met-Asn-Ser-Gln-Val
(neutral charge)

ΔDSE actin $^{+}H_3N$----------------Val
(1 positive charge)

The acidic residue sequence in the N-terminal segment of 4Ac actin is identical to that of rabbit skeletal muscle α-actin. Although the binding of S-1 to wild-type and 4Ac actin was similar to skeletal muscle α-actin, the catalytic efficiency of these actins in acto-S-1 ATPase varied over a 6-fold range (Cook *et al.*, 1993). The different roles of the N-terminal actin residues in the binding of S-1–ATP and the activation of S-1 ATPase, as is also evident from the work of Sutoh *et al.* (1991), suggest that these actin N-terminal residues may also differentially affect the binding and regulatory properties of caldesmon.

Effects of N-Terminal Charges on Caldesmon Inhibition of Acto-S-1 ATPase. We tested the involvement of the actin N-terminus in caldesmon function by examining caldesmon's ability to inhibit acto-S-1 ATPase activity in the complexes of S-1 with N-terminal yeast actin mutants. Figure 1 shows the relative acto-S-1 ATPase activities as a function of CDS/actin molar ratios. Full activity corresponds to acto-S-1 ATPase in the absence of caldesmon, and its absolute value varies for each actin type (see figure legend). Caldesmon inhibition of 4Ac and wild-type yeast actins is similar to that of rabbit α-actin, reaching a final decrease of 80% in the original acto-S-1 ATPase. The S-1 ATPase activities activated by DNEQ and ΔDSE yeast actins were regulated by caldesmon to a much smaller extent than wild-type or 4Ac actins. The inhibition for DNEQ and ΔDSE actins did not exceed 20% of the control ATPase activity.

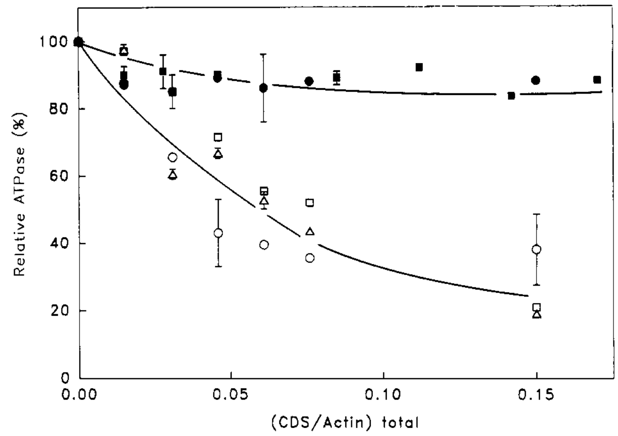

FIGURE 1: Effects of caldesmon on acto-S-1 ATPase of skeletal muscle and yeast actins. Acto-S-1 ATPase measurements were performed using the malachite green assay system. Symbols are as follows: ●, ΔDSE; ■, DNEQ; □, wild-type; △, α-actin; ○, 4Ac. ATPase is expressed as % control where 100% is the ATPase rate in the absence of caldesmon. The 100% ATPase rates varied for each actin as follows: α-actin, 5.35 s^{-1}; 4Ac, 1.8 s^{-1}; wild-type, 0.6 s^{-1}; DNEQ, 0.45 s^{-1}; ΔDSE, 0.10 s^{-1}. These values have been corrected for the ATPase rate of S-1 alone. The actin concentrations employed in these experiments were 4.0 μM for 4Ac, wild-type, and α-actins, 30 μM for DNEQ actin, and 7 μM for ΔDSE mutants. Error bars indicate the deviation between two sets of experiments.

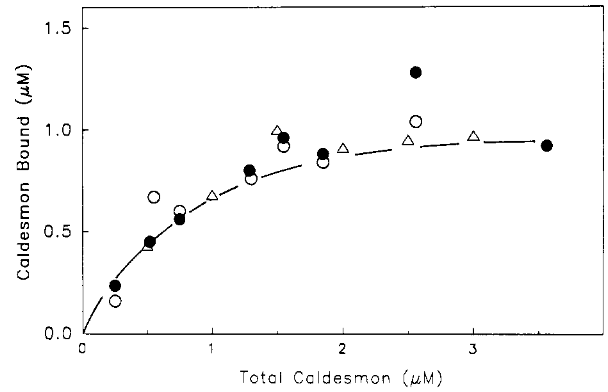

FIGURE 2: Binding of caldesmon to rabbit α-actin and yeast actins. The binding of caldesmon to actin was determined by cosedimentation of these proteins in a Beckman airfuge as described under Materials and Methods. 4 μM skeletal muscle α-actin (○), wild-type yeast actin (●), and DNEQ actin (△) were incubated with a range of caldesmon concentrations, as given on the abscissa. The molar amount of caldesmon bound to actin is given on the ordinate.

Measurements of acto-S-1 ATPase with DNEQ and ΔDSE actin mutants merit special caution. Since these mutant actins activate the myosin ATPase to only a small extent (Cook *et al.*, 1992), a concern can be raised about the reliability of these measurements. The simplest way to circumvent such a difficulty is to perform the ATPase inhibition measurements at much higher actin concentrations in order to increase the ATP turnover rate. This approach was feasible with DNEQ actin for which the ATPase measurements were made at 30 μM actin concentrations (Figure 1). Under these conditions, the turnover rate of DNEQ actin was increased to 0.45 s^{-1}, which is close to the turnover rate of wild-type actin, 0.60 s^{-1} (4 μM actin). The fact that the small inhibitory effect of caldesmon on DNEQ actin-activated S-1 ATPase was identical at 30 and 7 μM actin (not shown) adds credence to activity measurements at low actin concentrations. This conclusion is pertinent to the acto-S-1 ATPase measurements of ΔDSE actin, which cannot be accomplished at high actin concentrations due to the bundling of this actin mutant (Cook *et al.*, 1992). In further support of the ΔDSE actin-activated S-1 ATPase measurements, an increase in ionic strength of the assay medium from 10 to 50 mM KCl inhibited the low ATPase activities (data not shown).

Binding Experiments. It is possible that binding of caldesmon to actin and inhibition of acto-S-1 ATPase involve different sites on F-actin. To test this possibility, we measured the binding of caldesmon to the yeast wild-type and mutant actins. Results of cosedimentation experiments (Figure 2) show that α-, wild-type, and DNEQ actin bind caldesmon equally well over a range of CDS/actin molar ratios between

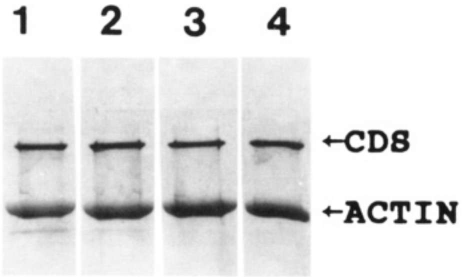

FIGURE 3: Binding of caldesmon to wild-type yeast actin and N-terminal yeast actin mutants. Cosedimentation of yeast actins (4 μM) and caldesmon (0.8 μM) was performed as described under Materials and Methods. Representative Coomassie-stained SDS gels of pelleted actin and CDS samples are shown in lanes 1–4; lane 1, wild-type actin; lane 2, 4Ac actin; lane 3, DNEQ actin; lane 4, ΔDSE actin. All lanes contain caldesmon.

0 and 0.90. Clearly, the differences in the primary structures of wild-type yeast and α-actins have no impact on the binding of caldesmon to F-actin. Furthermore, as shown by caldesmon binding to DNEQ yeast actin, substitutions at the N-terminus of actin have little, if any, effect on caldesmon binding (Figure 2). More limited cosedimentation experiments of caldesmon with all yeast actins support this conclusion. The binding of caldesmon to wild-type, 4Ac, DNEQ, and ΔDSE actin mutants was similar at the four CDS/actin molar ratios tested: 0.13, 0.20, 0.25, and 0.50. Figure 3 shows representative SDS gels of the pellets of wild-type, 4Ac, DNEQ, and ΔDSE yeast actins with caldesmon at a molar ratio of 0.20 for CDS/actin (data not shown for other CDS/actin molar ratios). Densitometric analysis of the pelleted fractions confirmed a similar binding of caldesmon to each actin, irrespective of its charge at the N-terminus. This is in striking contrast to the effect of the actin's N-terminal charge on the regulatory function of caldesmon (Figure 1).

Cosedimentation experiments were also performed with the rigor complexes of actin with S-1 and/or caldesmon. Under such conditions, S-1 displaces caldesmon from actin (Hemric & Chalovich, 1990). Indeed, for all actins used in this work, S-1 displaced 85% of caldesmon from the actin. This supports the conclusion that the N-terminal residues on actin cannot be crucial for the binding of caldesmon and S-1 to actin (data not shown). Therefore, a significant area of overlap between S-1 and caldesmon on actin must be outside the N-terminal region on actin. Clearly, caldesmon's contact sites outside of the N-terminal acidic sequence of actin [residues 18–28 (Patchell *et al.*, 1989) and the C-terminus of actin; e.g., Graceffa *et al.* (1991)] must allow for binding without much compromise in affinity. Weakening the interaction of one of caldesmon's contact sites is not sufficient to eliminate the binding.

Caldesmon Polymerization of N-Terminal Yeast Actin Mutants. Previous reports have shown that caldesmon induces the polymerization of G-actin (Galazkiewicz *et al.*, 1985, 1991) and the bundling of F-actin (Bretcher, 1984; Ngai & Walsh, 1984; Moody *et al.*, 1985; Dabrowska *et al.*, 1985). The myosin head, which shares binding sites with caldesmon on actin, also polymerizes G-actin (Cooke & Morales, 1971). In regard to polymerization studies, it is interesting that while mutations of the N-terminal region of actin have little effect on the binding of S-1 to F-actin, these mutations do substantially affect the binding of S-1 to G-actin (Chaussepied & Kasprzak, 1989; DasGupta *et al.*, 1990). To further compare S-1 and caldesmon interactions with actin, we tested the ability of caldesmon to polymerize N-terminal mutants of G-actin. Figure 4 shows the pyrene fluorescence of actin solutions as a function of time after the addition of caldesmon (1 μM). The polymerization curve of α-actin (Figure 4, curve b) is plotted for comparison to each of the other types of actin. The polymerization rate of α-actin (2.5×10^{-3} s^{-1}) is similar to that of 4Ac actin (3.1×10^{-3} s^{-1}, curve a). Wild-type yeast actin, with only two acidic residues, polymerized at a slower rate (7.9×10^{-4} s^{-1}, curve c). DNEQ and ΔDSE actins polymerized even more slowly (1.4×10^{-4} and 1.8×10^{-4} s^{-1}, respectively), although final measurements of the fluorescence intensity after 24 h indicated that both mutants polymerized to the same extent as the α-actin (see Table 1 for a summary of polymerization rates).

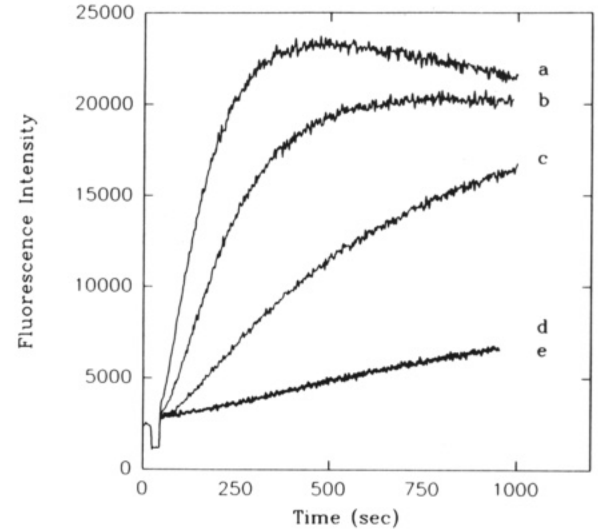

FIGURE 4: Caldesmon-induced polymerization of α-actin and yeast actins. 4 μM actin was mixed with 0.4 μM pyrene-labeled α-actin and monitored for polymerization by caldesmon. 1 μM caldesmon was added to induce the polymerization of actin. The progress of polymerization is reported by changes in fluorescence intensity with time: curve a, 4Ac actin; curve b, α-actin; curve c, wild-type actin; curve d, DNEQ actin; curve e, ΔDSE actin. The rates of polymerization are summarized in Table 1.

Table 1: Rates of Actin Polymerization by $MgCl_2$ and Caldesmon[a]

actin	polymerization rates (s^{-1}) $MgCl_2$	polymerization rates (s^{-1}) caldesmon
α-actin	1.9×10^{-3}	2.5×10^{-3}
4Ac actin	2.5×10^{-3}	3.1×10^{-3}
wild-type actin	2.5×10^{-3}	7.9×10^{-4}
DNEQ actin	2.4×10^{-3}	1.4×10^{-4}
ΔDSE actin	2.1×10^{-3}	1.8×10^{-4}

[a] These rates were calculated by dividing the initial slopes of the polymerization curves by the final equilibrium values of fluorescence upon the completion of polymerization.

The polymerization of actin was also monitored by light-scattering measurements in which the addition of labeled α-actin was unnecessary (data not shown). The rates of polymerization were identical to those determined by pyrene fluorescence.

As a control for the ability of actin to polymerize, the $MgCl_2$-induced polymerization of actin was also monitored for each of the yeast actins in both fluorescence and light-scattering measurements. $MgCl_2$ polymerization of the yeast actins was identical to that of α-actin, indicating that negative charges at the N-terminus or a deleted N-terminus was inconsequential to this reaction (Table 1). This suggests that the large differences in the polymerization of yeast actin mutants by caldesmon are related to their binding and not to an intrinsic polymerization defect in actin.

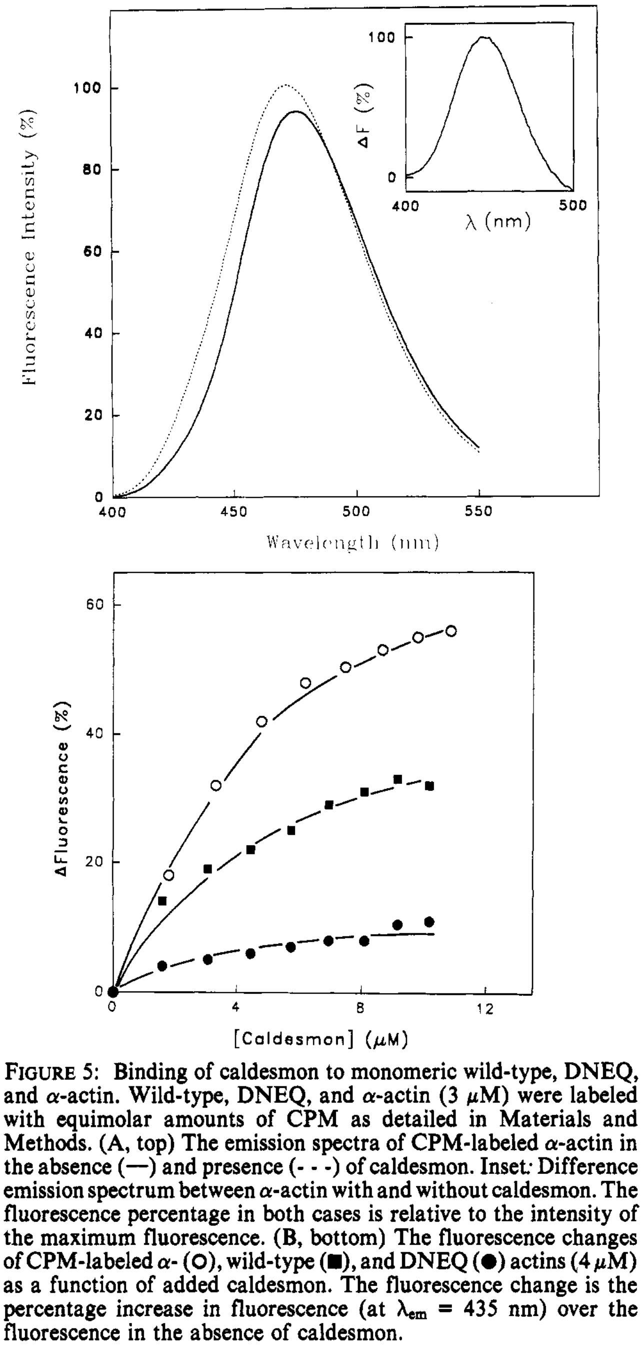

FIGURE 5: Binding of caldesmon to monomeric wild-type, DNEQ, and α-actin. Wild-type, DNEQ, and α-actin (3 μM) were labeled with equimolar amounts of CPM as detailed in Materials and Methods. (A, top) The emission spectra of CPM-labeled α-actin in the absence (—) and presence (- - -) of caldesmon. Inset: Difference emission spectrum between α-actin with and without caldesmon. The fluorescence percentage in both cases is relative to the intensity of the maximum fluorescence. (B, bottom) The fluorescence changes of CPM-labeled α- (○), wild-type (■), and DNEQ (●) actins (4 μM) as a function of added caldesmon. The fluorescence change is the percentage increase in fluorescence (at λ_{em} = 435 nm) over the fluorescence in the absence of caldesmon.

The binding of caldesmon to monomeric actin was assessed by fluorescence measurements. Skeletal α-actin, wild-type, and DNEQ actins were labeled with the fluorescent reagent (CPM) as described in Materials and Methods. A comparison of the emission spectra of CPM-labeled α-actin in the presence (Figure 5A, dashed curve) and absence (Figure 5A, solid curve) of caldesmon reveals a blue shift and an increase in fluorescence upon caldesmon binding to G-actin. The difference between the two emission spectra, presented in the inset to Figure 5A, was used in subsequent measurements to assess the binding of caldesmon to monomeric actin. Thus, we monitored the increase in fluorescence intensity of CPM-labeled actin at an emission wavelength of 435 nm upon the addition of caldesmon (0–10 μM) to α-, wild-type, and DNEQ actins (Figure 5B). Fluorescence measurements were recorded within seconds of caldesmon addition to insure that the intensity changes reflected caldesmon binding to monomeric actin.

Two control experiments showed that the polymerization of actin, if any should occur during the 20 or 30 s of the fluorescence measurement, would not complicate interpretation of the data. First, the emission spectrum of $MgCl_2$-polymerized CPM-labeled actin was identical to that of monomeric actin. Second, the binding of caldesmon to $MgCl_2$-polymerized CPM-labeled actin did not alter the emission spectra.

As shown in Figure 5B, the binding of caldesmon to DNEQ G-actin was greatly reduced compared with those of α-actin and wild-type actin. The binding of caldesmon to wild-type actin was reduced compared to that to α-actin. These results are consistent with the differences in the rates of caldesmon-induced polymerization of α-actin, wild-type, and DNEQ actins.

In analogy to the case with S-1, the contact between the N-terminus of G-actin and caldesmon is important to the binding of these proteins and, thus, the polymerization of actin by caldesmon. Although deletion of the N-terminus and the loss of its contribution to the binding of F-actin may be compensated for by other contact areas, such manipulation of actin greatly reduces G-actin interactions with caldesmon.

DISCUSSION

In this study, we show that neither changes in the charge density at the N-terminus of actin nor a deletion of three N-terminal residues has a significant effect on caldesmon binding to F-actin in the presence or absence of S-1. This result calls for a revision of the previous conclusion on the binding of caldesmon to actin's N-terminus. It also follows the pattern observed for acto-S-1 interactions. For both S-1–ATP and caldesmon, experiments with mutant actins reveal a smaller contribution of actin's N-terminus to the binding energy of these proteins than could be anticipated from cross-linking and other biochemical studies. Other sites on actin may either compensate for the loss of the N-terminal interaction or dominate the binding of S-1 and caldesmon to actin under some conditions. Caldesmon, thus, has multiple contact sites on actin that are able to compensate for a loss of one of the contact sites without a large loss in binding affinity. The locations of other caldesmon contact sites on actin are still unclear, although two regions including the C-terminus (Graceffa *et al.*, 1991; Crosbie *et al.*, 1991, 1992; Makuch *et al.*, 1992; Kolakowski *et al.*, 1992) and amino acids 18–28 [e.g., Adams *et al.* (1990)] have been implicated as components of the binding site. We expect that yeast actin mutants will be a powerful tool in the further elucidation of caldesmon binding sites on actin.

In contrast to the binding to F-actin, caldesmon inhibition of acto-S-1 ATPase was drastically influenced by a reduction in the number of the N-terminal acidic residues. Caldesmon inhibited the acto-S-1 activity of 4Ac, α-actin, and wild-type actin to the same extent (Figure 1). On the other hand, the acto-S-1 ATPase observed with DNEQ and ΔDSE actins resisted inhibition by caldesmon.

The nature of this effect merits some consideration. It is possible to view the acto-S-1 ATPase activity as consisting of two components: a basal activity that does not involve the actin N-terminus and an enhancement in this activity that does involve the N-terminal negative charges. According to such a mechanism, the small activation of myosin ATPase by DNEQ and ΔDSE actin mutants reflects the contribution of acto-S-1 contacts outside the N-terminal region of actin to the myosin ATPase. Caldesmon does not inhibit the remaining acto-S-1 activity of the deletion or neutral mutants. This would mean that acto-S-1 contacts related to the remaining ATPase do not overlap with caldesmon's sites on actin. An alternative second mechanism could involve the steering of caldesmon into its proper inhibitory configuration by the N-terminal region of actin without greatly affecting the affinity of caldesmon for actin. In both of these possible mechanisms, the N-terminal region of actin has important regulatory consequences, but it is less important in determining the affinity of binding. Also, both models are consistent with our earlier observations on the competition between S-1-ATP and caldesmon (Chalovich *et al.*, 1987; Velaz *et al.*, 1989; Marston & Redwood, 1992). This does not preclude the possibility of additional cooperative effects in the inhibition of ATPase activity. Such effects are likely to exist on the basis of the low amount of caldesmon required to inhibit ATPase activity, particularly in the presence of tropomyosin (Velaz *et al.*, 1989).

The localization of caldesmon in both stress fibers and ruffling membranes of cultured cells suggests that caldesmon participates in the motile events mediated by both actin and myosin interactions and reversible assemblies of actin filaments (Bretscher & Lynch, 1985). We found that the caldesmon-induced polymerization of yeast actins was strongly influenced by actin's N-terminal acidic residues. The reduced binding of caldesmon to the charge-deficient mutants indicates that the N-terminal residues of actin are critical for the binding of caldesmon to G-actin and the polymerization induced by it. This reduction in caldesmon binding to G-actin, in contrast with the binding to F-actin, highlights the distinct interactions of caldesmon with G- and F-actins. It is interesting that S-1, which also polymerizes actin, strongly depends on the N-terminus of actin for its binding to G-, but not F-actin. In analogy to S-1, the link between the N-terminal charges on G-actin and its polymerization by caldesmon result from charge-dependent binding of these proteins. Such binding may be initiated by the docking of caldesmon (or S-1) to the N-terminus on G-actin and followed by the establishment of other protein–protein contacts.

In summary, our results show that, although the N-terminal residues of actin do not contribute much to the binding of caldesmon to F-actin, they are critical for the interaction of caldesmon with G-actin. This work also shows that the N-terminal residues on actin are essential to the regulatory function of caldesmon.

REFERENCES

Adams, S., DasGupta, G., Chalovich, J. M., & Reisler, E. (1990) *J. Biol. Chem. 265*, 19652–7.

Aspenstrom, P., & Karlsson, R. (1991) *Eur. J. Biochem. 200*, 35–41.

Bartegi, A., Fattoum, A., Derancourt, J., & Kassab, R. (1990) *J. Biol. Chem. 265*, 15231–8.

Bertrand, R., Chaussepied, P., Audemard, E., & Kassab, R. (1989) *Eur. J. Biochem. 181*, 747–54.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–54.

Brenner, B., Yu, L. C., & Chalovich, J. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5739–43.

Bretcher, A. (1984) *J. Biol. Chem. 259*, 12873–80.

Bretcher, A., & Lynch, W. (1985) *J. Cell Biol. 100*, 1656–63.

Bryan, J., & Lee, R. (1991) *J. Muscle Res. Cell Motil. 12*, 372–5.

Chalovich, J. M. (1993) *Pharmacol. Ther. 55*, 95–148.

Chalovich, J. M., Cornelius, P., & Benson, C. E. (1987) *J. Biol. Chem. 262*, 5711–6.

Chaussepied, P., & Kasprzak, A. A. (1989) *J. Biol. Chem. 264*, 20752–9.

Cook, R. K., Sheff, D. R., & Rubenstein, P. A. (1991) *J. Biol. Chem. 266*, 16825–33.

Cook, R. K., Blake, W., & Rubenstein, P. A. (1992) *J. Biol. Chem. 267*, 9430–6.

Cook, R. K., Root, D., Miller, C., Reisler, E., & Rubenstein, P. A. (1993) *J. Biol. Chem. 268*, 2401–15.

Cooke, R., & Morales, M. F. (1971) *J. Mol. Biol. 60*, 240–61.

Cooper, J. A., Walker, S. B., & Pollard, T. D. (1983) *J. Muscle Res. Cell Motil. 4*, 253–62.

Crosbie, R., Adams, S., Chalovich, J. M., & Reisler, E. (1991) *J. Biol. Chem. 266*, 20001–6.

Crosbie, R. H., Chalovich, J. M., & Reisler, E. (1992) *Biochem. Biophys. Res. Commun. 184*, 239–45.

Dabrowska, R., Goch, A., Galazkiewicz, B., & Osinska, H. (1985) *Biochim. Biophys. Acta 842*, 70–5.

DasGupta, G., & Reisler, E. (1989) *J. Mol. Biol. 207*, 833–6.

DasGupta, G., & Reisler, E. (1992) *Biochemistry 31*, 1836–41.

DasGupta, G., White, J., Cheung, P., & Reisler, E. (1990) *Biochemistry 29*, 8503–8.

Galazkiewicz, B., Mossakowska, M., Osinska, H., & Dabrowska, R. (1985) *FEBS Lett. 184*, 144–9.

Galazkiewicz, B., Buss, F., Jockusch, B. M., & Dabrowska, R. (1991) *Eur. J. Biochem. 195*, 543–7.

Godfrey, J. E., & Harrington, W. F. (1970) *Biochemistry 9*, 886–93.

Grabarek, Z., & Gergely, J. (1987) *Biophys. J. 51*, 331a.

Graceffa, P., & Jancso, A. (1991) *J. Biol. Chem. 266*, 20305–10.

Haeberle, J. R., Trybus, K. M., Hemric, M. E., & Warshaw, D. M. (1992) *J. Biol. Chem. 267*, 23001–6.

Hemric, M. E., & Chalovich, J. M. (1988) *J. Biol. Chem. 261*, 16155–60.

Hemric, M. E., & Chalovich, J. M. (1991) *J. Biol. Chem. 265*, 19672–8.

Horiuchi, K. Y., Samuel, M., & Chacko, S. (1991) *Biochemistry 30*, 712–7.

Katsuyama, H., Wang, C.-L.A., & Morgan, K. G. (1992) *J. Biol. Chem. 267*, 14555–8.

Kodama, T., Fukui, K., & Kometani, K. (1986) *J. Biochem. 99*, 1465–72.

Kolakowski, J., Makuch, R., & Dabrowska, R. (1992) *FEBS Lett. 309*, 65–7.

Korn, E. D., & Hammer, H. A., III (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 23–45.

Kouyama, T., & Mikashi, K. (1981) *Eur. J. Biochem. 114*, 33–8.

Laemmli, U. K. (1970) *Nature 227*, 680–5.

Lash, J. A., Sellers, J. R., & Hathaway, D. R. (1986) *J. Biol. Chem. 261*, 16155–60.

Levine, A. B., Moir, A. J. G., & Perry, S. V. (1988) *Eur. J. Biochem. 172*, 389–97.

Lim, M., & Walsh, M. (1986) *Biochem. J. 238*, 523–30.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–75.

Makuch, R., Kolakowski, J., & Dabrowska, R. (1992) *FEBS Lett. 297*, 237–40.

Marston, S. B., & Lehman, W. (1985) *J. Biochem. 231*, 517–22.

Marston, S. B., & Redwood, C. S. (1991) *Biochem. J. 279*, 1–16.

Marston, S. B., & Redwood, C. S. (1992) *J. Biol. Chem. 267*, 16796–800.

Mejean, C., Boyer, M., Labbe, J. P., Marlier, L., Benjamin, Y., & Roustan, C. (1987) *Biochem. J. 244*, 571–577.

Miller, L., Kalnoski, M., Yunossi, Z., Bulinski, J. C., & Reisler, E. (1987) *Biochemistry 26*, 6064–70.

Miller, L., Phillips, M., & Reisler, E. (1988) *J. Biol. Chem. 263*, 1996–2002.

Moody, C. J., Marston, S. B., & Smith, C. W. J. (1985) *FEBS Lett. 191*, 107–12.

Ngai, P. K., & Walsh, M. P. (1984) *J. Biol. Chem. 259*, 13656–9.

Ngai, P. K., & Walsh, M. P. (1985) *Biochem. J. 230*, 695–707.

Nowak, E., Borovikov, Y. S., & Dabrowska, R. (1989) *Biochim. Biophys. Acta 999*, 289–92.

Patchell, V. B., Perry, S. V., Moir, A. J. G., Audemard, E., Mornet, D., & Levine, B. A. (1989) *Biochem. Soc. Trans 17*, 901.

Pfitzer, G., Fisher, W., & Chalovich, J. M. (1994) in *Mechanism of Myofilament Sliding in Muscle Contraction* (Sugi, H., Ed.), Plenum Publishing Corporation, Tokyo.

Sellers, J. R. (1991) *Curr. Opin. Cell Biol. 3*, 98–104.

Smith, C. W. J., & Marston, S. B. (1985) *FEBS Lett. 184*, 115–9.

Smith, C. W. J., Pritchard, K., & Marston, S. B. (1987) *J. Biol. Chem. 262*, 116–22.

Sobue, K., & Sellers, J. R. (1991) *J. Biol. Chem. 266*, 12115–8.

Sobue, K., Morimoto, K., Inue, M., Kaanda, K., & Kakiuchi, S. (1982) *Biomed. Res. 3*, 188–96.

Sobue, K., Takahashi, K., & Wakabayashi, I. (1985) *Biochem. Biophys. Res. Commun. 132*, 645–51.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–71.

Sutoh, K. (1982) *Biochemistry 21*, 3656–61.

Sutoh, K., Ando, M., Sutoh, K., & Toyoshima, Y. Y. (1991) *Proc. Nat. Acad. Sci. U.S.A. 88*, 7711–4.

Trayer, I., Trayer, H. R., & Levine, B. A. (1987) *Eur. J. Biochem. 164*, 259–66.

Velaz, L., Hemric, M. E., Benson, C. E., & Chalovich, J. M. (1989) *J. Biol. Chem. 264*, 9602–10.

Velaz, L., Ingraham, R. H., & Chalovich, J. M. (1990) *J. Biol. Chem. 265*, 2929–34.

Weeds, A., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–57.