Smooth Muscle Caldesmon Is an Extended Flexible Monomeric Protein in Solution That Can Readily Undergo Reversible Intra- and Intermolecular Sulfhydryl Cross-linking

A MECHANISM FOR CALDESMON'S F-ACTIN BUNDLING ACTIVITY*

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Caldesmon is a major F-actin binding protein of smooth muscle that has been implicated as a component of a thin filament regulatory system. Chicken gizzard caldesmon consists of polypeptides of Mr 135,000 and 140,000 which are closely related as determined by analysis of cyanogen bromide cleavage fragments. It is a highly extended flexible protein having a contour length of about 146 nm and a secondary structure composed primarily of random coil. Physical and chemical cross-linking data suggest that caldesmon exists as a monomer in solution. The cysteine content of caldesmon was determined to be 2 residues/poly peptide. Remarkably, in solution it readily undergoes sulfhydryl oxidation to form either an internal disulfide bridge in the protein or cross-links between individual polypeptides to form dimers, trimers, tetramers, etc. The internally cross-linked species have a smaller Stokes radius than the reduced molecules, indicating that the cross-link "trapped" the molecule in a compact conformation. Oxidized protein containing caldesmon oligomers is a potent F-actin bundling protein. Complete reduction of caldesmon abolishes the F-actin bundling activity. Since a vast excess of reducing agent is required to convert caldesmon from an oxidized to reduced state, it may exist in either state in vivo. Thus, the ability of caldesmon to undergo reversible sulfhydryl cross-linking, and thereby reversible F-actin cross-linking, may be of physiological significance.

Caldesmon inhibits the interaction of myosin with F-actin, and that this inhibition is alleviated by the presence of Ca²⁺ and calmodulin. Similar results have been obtained by other laboratories (4, 8). By contrast, Ngai and Walsh (9, 10) have indicated that pure caldesmon inhibits the actin-activated Mg²⁺-ATPase activity of smooth muscle myosin in the presence and absence of Ca²⁺-calmodulin. They have reported the existence in smooth muscle of a Ca²⁺-calmodulin-activated caldesmon kinase that they believe contaminated conventional caldesmon preparations. Moreover, they find that phosphorylated caldesmon has no effect on the myosin ATPase activity in a reconstituted contractile system. Thus the two sets of results indicate that caldesmon can regulate the actomyosin interaction, although the exact mechanism(s) remains to be clarified. The finding that caldesmon immunoreactive species are found in all smooth and non-muscle cells so far examined (11-14) indicates that caldesmon may be widely used to regulate contractile elements. Moreover, the finding that caldesmon is localized in smooth and non-muscle cell stress fibers in a striated pattern coincident with tropomyosin, myosin, and myosin light chain kinase, adds further evidence for a possible regulatory role for caldesmon (13).

There exist two discrepancies concerning the properties of smooth muscle caldesmon. The first relates to its subunit composition and shape. Sobue et al. (1, 17) reported that it is a globular heterodimer in solution, whereas our preliminary data indicated that it is a highly extended monomeric protein in solution (2). Second, several laboratories have reported that caldesmon can be a potent F-actin bundling protein (2, 15, 16), whereas Sobue et al. (17) have reported that it is not.

In this report we examine many physical and chemical properties of caldesmon and show that it is a remarkable molecule with some unusual properties that may relate to its physiological role. We also address the two discrepancies cited above. Our data indicate that caldesmon is a long, highly flexible monomeric protein in solution that can readily undergo intra- and intermolecular disulfide bond formation under oxidizing conditions. This disulfide bond formation leads to the formation of molecular adducts separable by physical techniques. Finally, we show that fully reduced caldesmon is not a potent F-actin bundling protein and that bundling activity is correlated with the appearance of disulfide cross-linked caldesmon species. The possible physiological role of disulfide bridging is discussed.

MATERIALS AND METHODS

Protein Purification—Chicken gizzard smooth muscle caldesmon was prepared from fresh material as described (3). Rabbit skeletal muscle actin was purified according to Spudich and Watt (18) and subjected to gel filtration on Sephadex G-150 in G buffer before use.

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Caldesmon Properties and Sulphhydryl Cross-linking

RESULTS

Physical Properties of Smooth Muscle Caldesmon—Caldesmon was purified as previously described (2) with modifications to reduce proteolysis and to ensure that the protein was isolated in a fully reduced form (3). As reported previously (2), smooth muscle caldesmon exists as two polypeptide chains of apparent molecular weights of 140,000 and 135,000.

The homogeneity of the preparation was analyzed by velocity sedimentation in a Beckman Model E ultracentrifuge. A single symmetrical peak, indicative of an homogeneous protein, was found having a sedimentation coefficient 2.54 S. This value is in line with an earlier estimate of 2.7 S, which was determined in the preparative centrifugation on the form with standard proteins (2). Reduced caldesmon was also subjected to molecular sieve chromatography on either a Sephacryl S-400 column or on an analytical Superose 6 fast protein liquid chromatography column and its elution profile compared with proteins of known Stokes radii. The protein eluted as a symmetrical peak and confirmed our earlier estimate of a Stokes radius of 91 Å (2). These two physical characteristics indicate that the protein is highly asymmetric. This prediction was verified by observing the molecules by electron microscopy after negative staining and low angle rotary shadowing (Fig. 1). Negatively stained preparations indicated a long flexible molecule (panel A), although the micrographs were not of sufficient clarity to allow a satisfactory length measurement. However, a width of about 3 nm was estimated for these molecules. Low angle rotary shadow images (panels B and C) of smooth muscle caldesmon also indicated an elongated molecule. The gallery of shadowed molecules presented in panel C indicates the great flexibility of this molecule. Contour measurements from shadowed images are presented in the form of a histogram in panel D. Under reducing conditions caldesmon has an average contour length of 146 ± 40 nm. A rather large range in the length of the molecule was recorded, probably because of the highly flexible nature of the molecule.

Circular dichroism spectra of purified caldesmon contained spectral minima of −63 and −57 × 10⁻² degrees cm² dmol⁻¹ at 203 and 221 nm, respectively. This indicated that its secondary structure is about 10% α-helix, 20% β-pleated sheet, and 70% random coil. These figures were the same when calculated either from the known spectra of configurations of poly-L-lysine at different pH values, or from the spectra of a group of proteins (ribonuclease, lysozyme, and myoglobin) whose secondary structure is known from x-ray crystallography (32).

Chemical Properties of Smooth Muscle Caldesmon—For amino acid analysis, the purified caldesmon was first subjected to Ca²⁺-dependent affinity chromatography on immobilized calmodulin to ensure homogeneity of the protein sample. The resulting analysis is shown in Table I and indicates the molecule is very rich in lysine, arginine, and glutamic/glutamine residues. The nitrogen content was determined to be 20.21%, which would indicate that about half of the total aspartic/asparagine and glutamic/glutamine residues exist in the acid form. Caldesmon also appears to be low in histidine, tyrosine, and cysteine. These and other chemical determinations indicate that caldesmon contains 2 cysteine residues/\( M_r - 140,000 \). The partial specific volume of caldesmon calculated from the amino acid analysis (24) is 0.72 cm³/g.

The absorption coefficient for freshly prepared caldesmon, based on quantitative amino acid analysis, was determined to be \( E_{280}^{1%} = 4.9 \) (Table I). A similar absorption coefficient was obtained independently by dissolving a known mass of lyophilized caldesmon in a high salt buffer. The somewhat low

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1. The abbreviations used are: EGTA, ethyleneglycol-bis(ethyleneenitrilo)tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).
Caldesmon Properties and Sulfhydryl Cross-linking

TABLE I

| Amino acid | Mole percent | Residues per 140,000 |
|------------|--------------|----------------------|
| Asx        | 6.2          | 76.4                 |
| Glx        | 28.0         | 345.1                |
| Ser        | 5.4          | 66.5                 |
| Gly        | 4.4          | 54.2                 |
| His        | 0.16         | 2.0                  |
| Arg        | 10.4         | 128.2                |
| Thr        | 3.8          | 46.8                 |
| Ala        | 12.6         | 155.3                |
| Pro        | 3.6          | 44.4                 |
| Tyr        | 0.51         | 6.3                  |
| Val        | 4.2          | 51.8                 |
| Met        | 0.88         | 10.8                 |
| Cys        | 0.15         | 1.9                  |
| Ile        | 1.2          | 14.8                 |
| Leu        | 4.4          | 54.2                 |
| Phe        | 1.0          | 12.3                 |
| Lys        | 12.9         | 159.0                |
| Trp        | ND*          |                      |

* ND, not determined.

absorption coefficient reflects the low content of aromatic residues in caldesmon.

The primary sequence relationship between the $M_r$-135,000 and 140,000 species was explored by comparison of peptides generated after cleavage at methionine residues with cyanogen bromide (Fig. 2). A very similar cleavage pattern was seen, but with a significant number of polypeptides from the 140,000 species having a slightly higher apparent molecular weight than those from the $M_r$-135,000 species. This indicates that the two polypeptides share considerable primary sequence homology and that the molecular weight heterogeneity may exist at a unique site in the molecule.

Subunit Structure of Caldesmon: Cross-linking Studies—Chemical cross-linking has been used extensively to investigate the subunit composition of protein complexes. A specific form of chemical cross-linking, oxidation of adjacent sulfhydryl residues to form covalent disulfide bonds, has frequently been used to demonstrate subunit associations and protein-protein associations in macromolecular assemblies. We therefore explored the effect of removing reducing agent from caldesmon followed by CuSO$_4$-o-phenanthroline-catalyzed oxidation. Analysis of the products of oxidation by SDS-PAGE in the absence of reducing agent revealed in all cases higher molecular weight oligomers with apparently no upper limit (Fig. 3, panel A). Although higher molecular weight oligomers were seen after simply removing the reducing agent, the degree of conversion to these higher species was clearly related to the amount of oxidizing agent added. Discrete species with apparent molecular weights of about 155,000, 160,000, 300,000, 450,000, and up (labeled “155K,” “160K”, CD$_{155}$, CD$_{160}$, etc. in the figure) can be resolved. A similar experiment conducted after modification of free sulfhydryls with p-chloromercuribenzenesulfonate greatly reduced the degree of formation of the higher species (not shown). This result implicates the sulfhydryls in the cross-linking reaction.

To explore whether the higher molecular weight cross-linked species were occurring between 135,000 and 140,000 polypeptides in a stable complex or due to random molecular collisions, we examined the concentration dependence of their formation (Fig. 3, panel B). The 155,000 and 165,000 species appeared in a concentration-independent manner, whereas...
the formation of the CD₂, CD₃, etc. species occurred in a concentration-dependent manner. To investigate from which monomeric species (135,000 or 140,000) the higher molecular weight species were derived, and to ensure that the cross-linking occurred between sulfhydryls, a gel slice containing the separated species was reduced and run in an orthogonal direction in a reducing gel (Fig. 3, panel C). This showed that the 155,000 species yielded only 135,000 and the 160,000 species yielded only 140,000, and that all the other higher species gave rise to both 135,000 and 140,000 polypeptides. All of these data support the concept that the 155,000 and 160,000 species result from intramolecular cross-links and that the CD₂, CD₃, etc. species result from cross-links between polypeptides colliding randomly, rather than between sub-units of a stable complex. This idea is corroborated by our inability to cross-link caldesmon with the bifunctional lysine-specific 6-Å cross-linker disuccinimidyldi tarte, although the cross-linker clearly reacts with caldesmon as indicated by the introduction of fuzziness into the caldesmon bands (not shown).

The physical size of the various cross-linked species was examined by gel filtration on Sephacryl S-400 in the absence of a reducing agent. Analysis of the resulting fractions on nonreducing gels (Fig. 4, panel B) showed that for the CD₂, CD₃, etc. species a clear correlation exists between their apparent Stokes radius and an increased apparent molecular weight on SDS-PAGE. When the cross-linked material was reduced with excess DTT prior to gel filtration, it ran as an homogeneous protein with an apparent Stokes radius of 91 Å (Fig. 4, panel A), the same as caldesmon that had never been oxidized (2). This demonstrates both the reversibility of the cross-linking, and that the oxidized species can easily be restored to the reduced monomers by reduction.

The 155,000 and 160,000 oxidized species are of particular interest. They have a slightly smaller apparent Stokes radius than reduced caldesmon, indicating a smaller physical size (Fig. 4). These data are consistent with the species containing intramolecular cross-links in single 135,000 and 140,000 caldesmon polypeptides to generate more compact molecules that are physically smaller but run with a higher apparent molecular weight on SDS-PAGE. An analogous situation in relative mobility in polyacrylamide gels is seen with DNA, where linear molecules migrate with a higher mobility than circular molecules of the same molecular weight.

Interaction of the Reduced and Cross-linked Species with F-actin—Caldesmon has been found to be a tight F-actin binding protein (1, 2, 6, 16, 17). It has been shown to increase the apparent viscosity of F-actin, both in an Ostwald viscometer (1) and using the falling ball viscometer (2, 17). Both reports have indicated that caldesmon is a potent F-actin bundling protein based on massive bundles seen in the light and electron microscopes (2, 15, 16), whereas others have reported that caldesmon is not an F-actin bundling protein (17). Based on the ability of caldesmon to cross-link to itself to form dimers, trimers, etc., we explored whether these apparent discrepancies could simply be a result of differences in the degree of caldesmon-caldesmon cross-linking. The first question we examined was under what experimental conditions caldesmon could undergo sulfhydryl cross-linking in the absence of catalysts, and what conditions would reverse it?

Initial experiments revealed that if caldesmon is simply dialyzed into a buffer containing little or no reducing agent, the protein is extensively oxidized into higher molecular weight species. Thus, if 3 μM caldesmon, prepared in the presence of 1 mM DTT, is dialyzed overnight into a degassed buffer containing no reducing agent, covalent cross-links are introduced between caldesmon polypeptides (Fig. 5, panel A). Examination of the air-oxidized species in the electron microscope revealed much larger molecules than in a reduced preparation, with molecules up to 500 nm being observed (Fig. 5, panel B). A titration of the amount of reducing agent required to reduce the sulfhydryl cross-links indicated that a vast molar excess (>0.5 mM DTT) was required for full reduction (Fig. 5, panel A). This is surprising since caldesmon has very few cysteine residues (see above). Addition of 0.5 mM KC or 5 mM EDTA had no detectable effect on the degree of oxidation. Only high levels of reducing agents or the sulfhydryl inhibitor p-chloromercuribenzenesulfonate prevented oxidation. Even prior treatment of reduced caldesmon with an excess of N-ethylmaleimide did not prevent all oxidation from occurring.

To examine the effect of sulfhydryl cross-linking on the bundling activity of caldesmon, 1 μM oxidized caldesmon and 10 μM F-actin were mixed and the resulting preparation examined by electron microscopy. Massive bundles of F-actin filaments were observed (Fig. 6, panel A). If increasing amounts of the reducing agent were added to the caldesmon and F-actin prior to mixing, bundles were still observed up to...
the addition of 0.1 mM DTT (panel B). However, at 1.0 mM DTT and above, no massive bundles of filaments were seen (panel C). F-actin alone in the absence of a reducing agent showed no tendency to form bundles (panel D). Analysis of the cross-linked state of the caldesmon in the presence of various concentrations of DTT (Fig. 6, panel A), revealed an excellent correlation between the absence of bundle formation and reduction of the cross-linked caldesmon species.

To explore whether caldesmon has F-actin cross-linking activity, as opposed to just bundling activity, we tested the ability of caldesmon to cross-link F-actin into structures that could be sedimented by low speed centrifugation, as is the case for filamin (25). Oxidized caldesmon was mixed with F-actin in a total of 250 µl and four otherwise identical aliquots made 0, 0.1, 1.0, and 10 mM in DTT. After half an hour incubation, the samples were subjected to 10,000 × g for 10 min and the protein composition in the top 50 µl determined by SDS-PAGE. In the absence of the reducing agent much of the caldesmon and actin was depleted from the top, whereas in the presence of >1.0 mM DTT no significant depletion
FIG. 5. Air oxidation of caldesmon. Panel A, nonreducing 5-10% SDS-PAGE of caldesmon after overnight dialysis into a buffer lacking a reducing agent, followed by treatment of the samples with various concentrations of DTT prior to electrophoresis. Lane 1, no added reducing agent; lanes 2-7, 0.05, 0.1, 0.5, 1, 5, and 10 mM DTT. Panel B shows a low angle rotary shadow micrograph of an oxidized sample of caldesmon. The small arrows indicate molecules of about 150 nm that may represent monomers, and the large arrows molecules that probably represent larger cross-linked species. Magnification: 27,000 X.

FIG. 6. Electron micrographs of the interaction of oxidized and reduced caldesmon with F-actin. Air oxidized caldesmon and F-actin were mixed in the presence of no DTT (panel A), 0.1 mM DTT (panel B), and 1.0 mM DTT (panel C) and observed after negative staining. Reducing agent, where applicable, was added to the proteins before mixing. Panel D shows F-actin alone in the absence of a reducing agent. All micrographs are at the same final magnification: 48,000 X.

occurred (Fig. 7). This indicates that the cross-linking activity of caldesmon, as measured by its ability to induce sedimentable F-actin containing structures, is only present in oxidized, and not reduced, samples. Taken together, the results strongly indicate that the potent F-actin bundling activity of caldesmon is a direct result of sulfhydryl cross-linking of caldesmon into higher oligomeric species and that reduced caldesmon itself is not a significant F-actin cross-linking protein.

DISCUSSION

In this report we have evaluated several physical and chemical properties of chicken gizzard smooth muscle caldesmon and explored further its in vitro F-actin bundling activity.

The physical data confirm our original report suggesting that caldesmon exists as an extended molecule in solution (2). Electron microscopy of caldesmon revealed a highly flexible extended morphology with an average contour length of about 146 nm and a thickness of 3 nm. This length is in close agreement with the recent shadowing data of Furst et al. (34) on caldesmon isolated from hog stomach smooth muscle, who reported a length of 140 nm. However, because this group also found molecules of shorter length (70 nm), they concluded that a contour length of 140 nm probably represented a dimer. We cannot immediately reconcile our results with theirs.
However, it must be pointed out that caldesmon is very susceptible to proteolysis, giving rise to fragmented molecules. This may be a particularly acute problem because, according to Furst et al. (34), only a small amount of the protein in the samples attaches to the support surface, thereby enriching for certain populations. It is also possible that such a flexible thin molecule may fold back on itself and have an apparently shorter length. It should be mentioned that 140 nm is not the longest molecule we have seen: in oxidized samples, where caldesmon-caldesmon disulfide cross-linked oligomers have been generated, much longer molecules have been observed (up to 500 nm). Although we currently favor a monomeric contour length of 140 nm, this question needs to be explored further.

The circular dichroism spectrum of caldesmon indicates that the highly extended conformation can be accounted for by a secondary structure containing primarily random coil. This is in sharp contrast to tropomyosin, another heat-stable elongated, but rigid protein, whose secondary structure is almost entirely made up of $\alpha$-helix (35). Other elongated high molecular weight proteins such as myosin and spectrin contain high levels of $\alpha$-helix (57% and 70%, respectively (36, 37)). Filamin, however, another highly elongated high molecular weight actin-binding protein is similar to caldesmon in that it has about 50% each of $\beta$-pleated sheet and random coil and little $\alpha$-helix and is also a highly flexible protein (38). The large amount of random coil in caldesmon may account for its highly flexible nature.

Chemical cross-linking, using either bifunctional cross-linking reagents or oxidation of adjacent cysteine residues to form disulfide bridges, has been used extensively to examine subunit structures of protein complexes. Sulphydryl cross-linking has been used, for example, to show that the two subunits of tropomyosin are in register (26, 39, 40), that the F-actin bundling protein band 4.9 of the erythrocyte cytoskeleton is a trimer (41), and to explore protein-protein interactions in the erythrocyte cytoskeleton (42, 43). Using a similar approach, we examined the effect of sulphydryl cross-linking on caldesmon and found that it was cross-linked into a number of distinct species. However, chemical cross-linking with bifunctional reagents showed no evidence for stable caldesmon-caldesmon interactions. Analysis of the disulfide cross-linked species by gel filtration separated the cross-linked species from the monomeric 135,000 and 140,000 poly-peptides. This clearly indicates that the oxidation has “captured” transient interactions between caldesmon molecules. The appearance of a ladder of species indicates that caldesmon must have at least two sulphydryls, which is in agreement with our chemical analysis. Indeed, at the level to which we can resolve the cross-linked species by SDS-PAGE, the apparent homogeneity of each species and the ratio of dimers to trimers to tetramers, etc., supports the low number of sulphydryls as determined by amino acid analysis. This is in contrast to the report of Ngai and Walsh (10) who reported that gizzard caldesmon, isolated by a different procedure, contains 6 cysteine residues. Recent results employing chemical cleavage at cysteine residues are consistent with gizzard caldesmon containing only 2 cysteine residues. The low number of sulphydryls makes the oxidation during removal of reducing agent by dialysis all the more remarkable, considering that sulphydryls are only very slowly reactive to molecular oxygen in the absence of an additional oxidizing agent. The ease with which we were able to generate oligomers provides a cautionary tale of how the generation of sulphydryl cross-linked species as detected simply by SDS-PAGE, but without an analysis of the native size of the cross-linked products, could lead to incorrect conclusions about the quaternary structure of a protein.

Both our earlier (2) and the present physical data suggest that reduced chicken gizzard caldesmon exists as a monomer in solution. In addition, a close inspection of the gel filtration data on oxidized caldesmon provides supporting evidence that reduced caldesmon is a monomer in solution. In particular, if reduced caldesmon were a dimer in solution, different results would be predicted. A cross-link between subunits in the dimer would migrate in the gel filtration column exactly as the reduced molecule but show up on nonreducing SDS-PAGE as a dimer; this was not found. A cross-link between two dimeric molecules to give a tetramer with a single disulfide cross-link would give a larger sized molecule on gel filtration, but yield two monomers and a dimer on nonreducing SDS-PAGE; this also was not seen. The results of the gel filtration of the sulphydryl cross-linked species strongly suggest that reduced caldesmon exists as a monomer in solution.

Sobue et al. (1, 7, 17) have reported that caldesmon is a globular heterodimer in solution. This conclusion appears to be based solely on gel filtration data and reducing SDS-PAGE.

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2 W. P. Lynch, V. M. Riseman, and A. Bretscher, unpublished data.
It is not clear how to interpret their results as they failed to take into consideration the shapes of molecules, as they used, for example, monomeric and dimeric filamin as globular molecular weight standards (17). Our physical data, and that of others (94), clearly indicate that caldesmon is not globular but a highly extended molecule. Purst et al. (34) also reported that caldesmon can exist as a heterodimer in solution based upon gel filtration data. However, since these authors were not aware of the ease of solution sulfhydryl cross-linking, it is likely that they were detecting sulfhydryl cross-linked dimers. In our experiments using a vast excess of reducing agent, we have never detected a tendency of caldesmon to oligomerize.

The 155,000 and 160,000 species seen in oxidized preparations of caldesmon are of notable interest. Upon reduction, the 160,000 band yields only the 140,000 species, and the 155,000 band yields only the 135,000 species. The 155,000 and 160,000 species have a smaller Stokes radius than reduced caldesmon, indicating a more compact molecule. This finding and the larger apparent molecular weight on SDS-PAGE would be consistent with these species being monomers of caldesmon with an internal disulfide bridge between distant sulfhydryls. An analogous shift in mobility in polyacrylamide gels is seen for DNA, where linear molecules migrate faster than circular molecules of the same molecular weight. The ability of caldesmon to form an intramolecular disulfide bridge and hold the molecule in a more compact conformation attests to the flexibility of the molecule in solution. As far as we are aware, this is the first example of a disulfide bond capturing a native flexible molecule in one of its conformations to give a structure with hydrodynamic properties different from its parent molecule.

We have explored whether in vitro oxidation of sulfhydryls could be responsible for the potent F-actin bundling activity reported for caldesmon (2, 15, 16) and found that oxidized caldesmon is an F-actin bundling protein whereas fully reduced caldesmon is not. Presumably the oxidation of caldesmon into dimers, trimers, etc. generates molecules with two or more F-actin binding sites and endows the resulting molecules with the F-actin bundling activity. Sobue et al. (17) have claimed that the bundling activity of caldesmon is due to freeze-thawing samples to form caldesmon aggregates with bundling activity. However, nonspecific aggregation could not account for the highly ordered structural arrays seen in thin sectioned preparations of caldesmon-induced F-actin bundles (2). The regular spacing between F-actin filaments in these arrays result from specific cross-linking by oligomers generated by disulfide bonds between individual monomers. In addition, we have shown, using fresh material, that controlled oxidation can induce bundling activity, and that this activity is reversed by reduction of caldesmon to monomers.

Two further aspects of F-actin cross-linking are of importance. First, although the potent F-actin bundling activity is clearly related to the formation of oligomeric forms of caldesmon, does fully reduced monomeric caldesmon have some F-actin cross-linking activity? Our experiments using electron microscopy and low speed centrifugation suggest that fully reduced caldesmon is not a significant F-actin cross-linking protein.

The second aspect is whether the ease of cross-linking caldesmon into oligomers, and hence F-actin bundling, is of physiological significance. It is interesting to note that preparations of caldesmons from chicken gizzard (2, 15, 16), hog stomach (34), and platelets (33) display various degrees of F-actin cross-linking suggesting that this phenomenon is probably not restricted to caldesmon from just one system. We have found that a vast nolar excess (at least 100-fold) of the reducing agent DTT is required to fully reduce disulfide bonds between monomeric caldesmon molecules. Considering that gizzard caldesmon is very low in cysteines, this is a surprising result. Although the environment in a cell is reducing, it is possible that monomeric caldesmon is in equilibrium with dimers, trimers, etc. through oxidation of sulfhydryls, or even that the state of oligomer formation is regulated by changes in the local reducing environment in the cell. This idea is consistent with the observation that ATP depletion of erythrocyte cytoskeletons can be disrupted by the addition of the sulfhydryl reagent p-chloromercuribenzenesulfonate (44) and the site of action of this reagent is suspected to be band 4.9, a potent F-actin bundling protein (41). The subunits of this protein have also been shown to undergo sulfhydryl cross-linking in the absence of catalyzed oxidation (41).

How do the properties of caldesmon relate to its physiological role? In vitro evidence indicates that, in conjunction with tropomyosin and calmodulin, it confers some Ca2+ regulation on filament thin filaments, and in situ localization data place it in a position appropriate for such a role (13). Proteolytic dissection studies have shown that caldesmon contains a M₄0,000 F-actin and calmodulin binding domain at one end of the molecule, and that the isolated domain is able to regulate the acto-myosin interaction in vitro (45). This raises the question of what the other ~100,000 of the molecule does, which is presumably a highly extended domain. It is conceivable that this portion binds to other caldesmon molecules, or to other molecules in the contractile apparatus. A possible role for this portion of the molecule might include further regulatory functions. Alternatively, this part of the molecule may be a "latch" that maintains tension after contraction, a possibility for caldesmon that has been suggested previously (5).

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