Human coflin possesses the tendency for self-association, as indicated by the rapid formation of dimers and oligomers when reacted with water-soluble carbodiimide, Ellman’s reagent, or glutathione disulfide. Intramolecular disulfide bonds involve Cys\(^{89}\) and probably Cys\(^{147}\) of two adjacent coflin units. The disulfide-linked dimers and oligomers exhibit a biological activity distinct from the monomer. While monomeric coflin decreased viscosity and light-scattering of F-actin solutions, dimers and oligomers caused an increase in viscosity and light scattering. Electron microscopy revealed that coflin oligomers induce the formation of highly ordered actin bundles with occasionally blunt ends similar to actin-cofilin rods observed in cells under oxidative stress. Bundling activity of the disulfide-linked oligomers could be completely reversed into severing activity by dithiotreitol. Formation of coflin oligomers occurred also in the presence of actin at pH 8, but not at pH 6.8, and was significantly enhanced in the presence of phosphatidylinositol 4,5-bisphosphate. Our data are consistent with the idea that coflin exists in two forms in vivo also as monomers exhibiting the known severing activity and as oligomers exhibiting actin bundling activity. However, stabilization of coflin oligomers in cytoplasm is probably achieved not by disulfide bonds but by a local increase in coflin concentration and/or binding of regulatory proteins.

The actin cytoskeleton plays an essential role in many cellular processes such as cytokinesis, changing of cell shape, endocytosis, and exocytosis. All of these processes are dependent on the correct spatial and temporal organization of the actin cytoskeleton (i.e. its regulated polymerization and depolymerization). These reorganizations of the actin cytoskeleton are regulated by a variety of actin-binding proteins, which in turn are regulated by external stimuli such as Ca\(^{2+}\) levels, phosphoinositides, pH, or reversible phosphorylation (1–3).

The turnover rates of actin filaments in living cells (reviewed in Refs. 4 and 5) are regulated by external stimuli such as Ca\(^{2+}\) and/or binding of regulatory proteins. This discrepancy is mainly due to the activity of ADF/cofilins (AC), which are able to increase the filament turnover in vitro up to levels found in living cells (6).

The essential role of AC in enhancing the turnover of actin filaments has been demonstrated in Listeria motility assays (6, 7) and in living yeast cells (8).

The AC are a class of small (13–19 kDa) actin-binding proteins, which are ubiquitous in eukaryotes (reviewed in Refs. 4 and 5). They typically localize to regions of rapid actin assembly like neural growth cones (10), developing skeletal muscle (11), cell cortex (12, 13), ruffling membranes (14), and cleavage furrow (15, 16). AC have been shown to be essential for the viability of Saccharomyces cerevisiae (12, 16), Dictostelium discoideum (17), Caenorhabditis elegans (18), Xenopus laevis (13), and Drosophila melanogaster (19). In contrast to other actin-binding proteins, AC are able to form 1:1 complexes with both G- and F-actin (20), exhibiting a preference for ADP-actin over ATP-actin (6). The mechanism by which AC depolymerize actin filaments was long controversial. Earlier studies suggested that depolymerization of actin filaments was the result of severing (21, 22), thereby increasing the number of filament ends. This view was challenged when it was shown that AC increase the off-rate for actin subunits at the pointed end of the actin filament (6). The two mechanisms are not mutually exclusive (23), and recently a mutagenic study revealed that both mechanisms take place side by side and can be uncoupled (24).

The activity of AC is regulated in various ways. Best characterized is the reversible phosphorylation of a conserved serine residue at the amino terminus (except for yeast and Dictyostelium, where no phosphorylated form of coflin was found) (25–27). In vertebrates, phosphorylation leads to the inhibition of actin binding in vitro (25) and to accumulation of actin filaments in vivo (28, 29). In vertebrates, phosphorylation is catalyzed through LIM kinase 1 or LIM kinase 2 (28, 29), which are regulated by the GTpases Rac and Rho/Cdc42, respectively (28–30). Regulation of AC thus depends on members of the Rho family GTpases, which are known to regulate the formation of filopodia, lamellipodia, and stress fibers in vertebrate cells (31). Additionally, actin binding and depolymerizing activities of AC are regulated by binding of phosphoinositides phosphatidylinositol 1,4,5-monophosphate and PIP\(_2\), to the actin binding sites of AC, a process that inhibits AC activity (32–34). Early studies proposed that depolymerization and severing of F-actin by AC is dependent on pH, with more extensive depolymerization at pH 8 than at pH \(< 7\) (22, 35, 36). The pH effect depends on the AC protein used, as concluded from more recent studies showing that severing by AC is independent of pH, whereas the depolymerizing factor Aip1, actin-interacting protein 1; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-diethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; N\(_2\)H; 2-nitro-5-thiobenzoate; PIP\(_{2}\), phosphatidylinositol 4,5-bisphosphate; RBS(CH\(_2\))\(_6\)SSR \((n = 3, 5, 6, 7, 8, 9, 10, and 12)\); alkylenebis(5,5-dithio-2-nitrobenzoic acid); MOPS, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography; RP-HPLC, reverse-phase HPLC; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
critical concentration of the AC-actin complex is increased at higher pH values, an effect that leads to an increase in unpolymerized actin (6, 37).

Binding of human cofilin to actin filaments changes the filament twist by 5° per unit, resulting in a reduced crossover length (38). This structural change prevents binding of tropomysin (39) or of the mushroom toxin phallolidin (40), suggesting that binding of AC and other molecules to actin filaments can be mutually exclusive. In Xenopus (41) and yeast (42, 43), the actin-interacting protein 1 (Aip1) was recently shown to bind to both actin and cofilin to enhance the filament disassembly activity of cofilin in vitro. Furthermore, Aip1 is required to restrict cofilin localization to yeast cortical patches, whereas in the absence of Aip1, cofilin is found in actin cables as well (42).

The original aim of the present study was to identify contact sites of actin and cofilin by thiol-specific cross-linking reactions, using reagents experienced in our laboratory for cross-linking studies with myosin S1 (44). Interestingly, we found that not only the expected actin-cofilin conjugate was formed, but also dimers and oligomers of cofilin in considerable amounts. Self-association of cofilin also occurred in the absence of actin and thus indicated an intrinsic property of cofilin. Disulfide-linked oligomers and dimers were isolated and analyzed for their structure and function. The studies on the structure of the actin-cofilin complex will be presented in a forthcoming paper.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cross-linking reagents 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC), 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), and N,N'-p-phenylenediamine were purchased from Sigma. Bis-sulfosuccinimide, cystamine, Ellman's reagent, and reagents for cross-linking with Ellman's reagent were obtained from Pierce. Reverse-phase C_{18} minicolumns for peptide purification were from Millipore and used according to the manufacturer's instructions. Endoprotease GluC was purchased from Roche Molecular Biochemicals. PIP_{2} was purchased according to the manufacturer's instructions. Proteins used as standards consisting of a mixture of substance P, the fragment 1–40 of angiotensin, oxidized insulin B chain, and ubiquitin. Spectra were recorded and analyzed using the Kratos Kompackt software, version 5.2.0.

**Purification of Cross-linked Dimers and Oligomers**—Cross-linking of cofilin (100 μM) was carried out in 10 mM Tris-HCl, pH 8, for 3 h on ice, then on ice. Cross-linking of Cofilin Thiols Involved in Cross-linking—In order to label cofilin thiols not participating in the cross-link reaction, c-carboxynitrophenylsulfonyl fluoride, reactions were carried out as described above except for a 5-fold excess of Ellman's reagent. The labeled oligomers were purified by HPLC gel filtration chromatography using a Waters 600 HPLC system equipped with a Waters 996 photodiode array. Separation was performed at a flow rate of 0.5 ml/min on two TSK 3000-G SW columns (Amersham Biosciences; 7.5 × 600 mm) in series on 0.1 M Tris-HCl, 0.1 M NaCl, 0.1% sodium azide, pH 7. Usually, 200 μl of the reaction mixture was injected for a single run. Runs were monitored by following the absorbance at 214 and 280 nm, and collected fractions were identified on 12.5% SDS-PAGE. Oligomer, dimer, and monomer fractions were concentrated by ultrafiltration in Minicon (Amicon) concentrators in a Biofuge A (Haeraeus) at 4000 rpm and dialyzed overnight at 4 °C against the corresponding reaction buffer.
of 1 ml/min. Labeled peptides were identified by their absorption at 330 nm and analyzed by mass spectroscopy and Edman degradation.

**RESULTS**

**Formation of Cofilin Oligomers**—Reaction of recombinant human cofilin with the zero-length cross-linker Ellman’s reagent (5,5'-dithio-2-nitrobenzoic acid) leads to the formation of dimers, trimers, and higher oligomers of cofilin even at equimolar concentrations of cross-linker and protein (Fig. 1). The frequently used cross-linking reagent EDC achieved the formation of dimers as well, but higher oligomers were almost absent (Fig. 1). In addition, the carbodiimide reaction required a high excess of reagent and a prolonged reaction time. Oligomeric cofilin was likewise formed when thiol-specific cross-linking reagents was used, but they precipitated with reagents of 1 eq of Ellman’s reagent by HPLC gel filtration chromatography (Fig. 4) and analyzed the peak fractions by SDS-PAGE (Fig. 4, inset). Most of the protein material was recovered as a mixture of oligomers with n ≥ 4 (peak 1), followed by small peaks of trimer (peak 2) and dimer (peak 3). Next, a small amount of cofilin (peak 4) was eluted, which, although separated from the bulk of cofilin monomer (peak 5), behaved as a monomer in SDS-PAGE. This species could represent a noncovalently linked dimer (note that gel filtration experiments were carried out under nonnondenaturing conditions, while SDS-PAGE...
leads to denaturation of proteins) or a monomer with intramolecularly cross-linked thiol groups. Since the trimer (peak 2) was obtained with poor yield and contaminated with dimer, we confined the investigation of the biological properties to the dimer obtained with poor yield and contaminated with dimer, we concluded that F-actin had changed its state of aggregation (22). We confirmed this effect by capillary viscosimetry using the cofilin monomer taken from the HPLC fraction (peak 5). At the first arrow, 10 μM F-actin at pH 8 immediately decreased light scattering to values as estimated from comparison with the diameter of single filaments. After the addition of cofilin dimers and oligomers (Fig. 5b). In control experiments, the addition of cofilin monomer to F-actin at pH 8 immediately decreased light scattering to values as reported by others (22), reflecting the breakdown or shortening of filaments. In contrast, the addition of the cofilin dimer in equimolar amounts enhanced light scattering by a factor of 3. The oligomeric fraction of cofilin had an even more pronounced effect and increased light scattering by a factor of 30 (Fig. 5b).

Electron Microscopy Studies—From the strong increase in light scattering, especially upon the addition of cofilin oligomers, we concluded that F-actin had changed its state of aggregation. We tested this using electron microscopy and found that under the conditions employed for capillary viscosimetry and light scattering experiments actin filaments formed bundles when cofilin dimers or oligomers were added. A typical image obtained from negative staining experiments with F-actin and cofilin oligomers at pH 8 (Fig. 6A) showed a regular and longitudinal alignment of groups of 5–8 filaments as estimated from comparison with the diameter of single filaments.
Electron micrographs were obtained by negative staining and oligomers. A 1:1:1 caused an even more pronounced aggregation of actin monomers and oligomers added to filamentous actin at a ratio mixture of cofilin oligomers and monomers on filamentous actin. Cofilin occasionally blunt ends (Fig. 6, revealing the highly ordered structure of these bundles with were confirmed in experiments using electron cryomicroscopy, aments that were still present in the sample. These results exhibited actin bundling activity and not actin depolymerizing values (i.e. At lower pH values (i.e. pH 6.6), actin aggregates induced by cofilin oligomers appeared irregular and decorated with knoblike structures (Fig. 6D). Similar images were obtained upon the addition of dimers at pH 6.6 (data not shown). In a control experiment, cofilin monomers made actin filaments disappear and left only a few short filaments behind (data not shown). These data suggest that self-association of cofilin with 1 eq of Ellman's reagent in the presence of 'activity...—association of cofilin. In order to obtain information on the structure of the—phenomenon might exist in vivo, we also examined the effect of a mixture of cofilin oligomers and monomers on F-actin. Cofilin monomers and oligomers added to filamentous actin at a ratio of 1:1:1 caused an even more pronounced aggregation of actin filaments into bundles in that the bundles became broader and appeared almost like sheets composed of 50 or more single filaments (Fig. 7A). In corresponding experiments using pH 6.6 instead of pH 8, the bundles were less regular and packed more tightly. Additionally, they were decorated with knob-shaped structures presumably representing cofilin oligomers (Fig. 7B). Treatment of these samples with DTT made all aggregates disappear and left only a few short filaments behind (data not shown). In summary, these pictures provide evidence that cofilin oligomers and dimers, as such, and even more so in mixture with monomers, exhibit actin bundling activity, with the shape and structure of the actin bundles varying with the experimental conditions.

**Phosphoinositides Enhance the Oligomerization of Cofilin**—The phosphoinositide PIP$_2$ is known to bind to actin binding sites of cofilin, thereby inhibiting its binding to actin in vitro (32). Furthermore, PIP$_2$ is supposed to be a regulator of cofilin activity in vivo (48). We therefore performed the oligomerization of cofilin with 1 eq of Ellman's reagent in the presence of various concentrations of PIP$_2$ (0–10 eq) and found a significant increase in the yield of trimers and higher oligomers in the presence of the phosphoinositide (Fig. 8). This effect was highly significant, since already 1 eq of PIP$_2$ caused self-association into dimers and higher oligomers of ~90% of the whole cofilin present. This value appeared to increase further when PIP$_2$ was applied in excess. Enhancement of cofilin oligomerization depended on the PIP$_2$ concentration in a nonlinear manner, reaching a maximum at a molar ratio of lipid/protein of 5:1. The effect of PIP$_2$ was also observed in the presence of 0.15 M NaCl (data not shown). These data suggest that self-association of cofilin is strongly enhanced in the presence of PIP$_2$, and it is tempting to speculate that the known inhibition of actin depolymerizing activity of cofilin by binding of PIP$_2$ involves self-association of cofilin.

**Identification of Cofilin Thiols Involved in Oligomer Formation**—In order to obtain information on the structure of the cofilin oligomers, we tried to identify the cofilin thiol groups involved in the disulfide bridges. For this purpose, we performed cross-linking reactions with an excess of Ellman's reagent (3 eq), anticipating that exposed thiols not participating in the disulfide bridges would be tagged as mixed disulfides with NTB (2-nitro-5-thiobenzoate, cofilin-S-S-C$_6$H$_3$NO$_2$(COONa)). In previous work of our laboratory (49), mixed disulfides of this type were shown to be stable in the absence of reducing agents and easily identifiable by their absorption maximum at 330 nm. The oligomeric cofilin derivatives were isolated by HPLC gel filtration as described above. By fragmentation with endoprotease Glu-C and subsequent separation of the fragments by
FIG. 8. PIP$_2$ enhances the formation of cofilin oligomers. Cofilin (20 μM in 10 mM Tris-HCl, pH 8) was cross-linked with Ellman's reagent (20 μM; 1 h, 20 °C) in the presence of various molar ratios of PIP$_2$ (0–10 eq). Aliquots of the reaction mixtures were subjected to SDS-PAGE and visualized by silver staining.

RP-HPLC, we were able to obtain three major fractions exhibiting absorption at 330 nm (Fig. 9A). Two of them represented labeled cofilin peptides (Fig. 9A, peaks 1 and 2), whereas the third one (peak 3) consisted of free NTB. The two peptides were identified as fragments 55–90 and 135–141 of human cofilin by mass spectroscopy and amino-terminal sequencing, suggesting that Cys$^{80}$ and Cys$^{139}$ were labeled (i.e. not involved in the disulfide links of the oligomers). This suggests that the cofilin oligomers were not randomly linked but connected via disulfide bonds between Cys$^{39}$ and, most likely, Cys$^{147}$ of neighboring subunits.

Proof of the labeling of Cys$^{80}$ (i.e. the noninvolvement of this thiol) was given by MALDI-TOF analysis (Fig. 9B) and Edman degradation. Fragment 2 (peak 2) had a mass of 4289 Da and the amino-terminal sequence ILVG, attributable to the fragment 55–90 of human cofilin. After treatment with 2-mercaptoethanol, this fragment yielded a mass of 4163 Da, corresponding to the mercaptoethanol derivative of fragment 55–90 (i.e. (55–90)-S-S-CH$_2$-CH$_2$-OH) (Fig. 9B). It thus gave final proof of the noninvolvement of Cys$^{80}$ in intermolecular disulfides. The first fraction (peak 1) contained a mixture of three peptides as concluded from amino-terminal sequencing. One of these fragments could clearly be identified by its mass spectroscopy signal (1073 Da) and amino-terminal sequence (KLGG) as fragment 152–151 of human cofilin by Edman degradation and MALDI-TOF analysis (see Fig. 9B). Peak 3 in the 330-nm profile consisted of free NTB. B. MALDI-TOF analysis of peak 2 (Fig. 9A). Shown is an overlay of two MALDI-TOF spectra before and after treatment of peak 2 with 2-mercaptoethanol. The mass/charge ratios (m/z) 4163 and 4289 corresponded to the NTB-adduct (peak 1) and the 2-mercaptoethanol adduct (peak 2) of the fragment 55–90 of human cofilin, respectively.

FIG. 9. Identification of thiol groups involved in cofilin oligomer formation. a, reverse-phase HPLC peptide map of endoproteinase Glu-C digest of cofilin oligomers in which the thiols not involved in cross-linking were labeled by Ellman's reagent. Shown is an overlay of the elution profiles obtained at 214 nm (top) and 330 nm (bottom). Fractions comprising peaks 1 and 2 were collected and subjected to Edman degradation and MALDI-TOF analysis (see Fig. 9B). Peak 3 in the 330-nm profile consisted of free NTB. B. MALDI-TOF analysis of peak 2 (Fig. 9A). Shown is an overlay of two MALDI-TOF spectra before and after treatment of peak 2 with 2-mercaptoethanol. The mass/charge ratios (m/z) 4163 and 4289 corresponded to the NTB-adduct (peak 1) and the 2-mercaptoethanol adduct (peak 2) of the fragment 55–90 of human cofilin, respectively.

would not easily be detectable. We therefore stabilized the oligomers by disulfide cross-linking and, after separation of dimers, trimers, and oligomers from the monomer by gel filtration, studied the properties of the pure fractions in the absence of monomer. We found that oligomerization of cofilin not only inhibited the depolymerizing activity of cofilin but changed it into an actin-bundling activity. Since the majority of the studies so far report cofilin as an actin-depolymerizing and/or -severing protein, the equilibrium in a cofilin solution must indeed be toward the monomer. This may explain why bundling activity was not detected in the past. Autoxidation, a process that would stabilize cofilin oligomers by disulfide bonds, is unlikely to occur, since ADF/cofilin proteins are usually kept in buffers containing high concentrations of DTT (2–5 mM) (see, for example, Refs. 51 and 52). Only one study (38) reports that cofilin can cause the formation of short actin bundles in F-actin solutions. We suggest that in this case noncovalently associated cofilin had caused the effect. Independent of biological activity, the existence of a cofilin/cofilin interaction was recently detected in a yeast two-hybrid screen (42). The authors suggested that the interaction might occur by bridging of cofilin molecules through actin. We here provide evidence that cofilin dimers and
oligomers are formed independent of actin.

Covalently linked oligomers of cofilin were obtained either by reaction with EDC/NHS or with thiol-specific cross-linking reagents. Reaction with EDC/NHS led mainly to dimers and required a high concentration of cross-linking reagent, suggesting that, in the complex of cofilin units, carboxyl and amino groups are in positions unfavorable to form an amide bond. In contrast, thiol-specific cross-linkers like RSS(CL)_2(SSR or Ellman's reagent were suited even at equimolar reagent concentrations to form dimers and higher oligomers as well. From the high yield of zero-length cross-linking between cofilin molecules with Ellman's reagent, we conclude that cofilin exposes thiol groups, which in the oligomers come close enough to form a disulfide bridge (i.e. approach to a distance of ~2 Å). This oxidation reaction did not cause any irreversible denaturation of cofilin, as shown by the fact that reductive cleavage of the disulfide bonds by DTT ablished bundling activity and fully restored the known severing activity.

Formation of cofilin dimers and oligomers was not dependent on the high oxidation potential of Ellman's reagent but was possible with the physiological oxidant GSSG as well. Since the ratio of glutathione/GSSG in cytoplasm is ~100:1, it is unlikely that GSSG acts as an oxidizing agent for stabilizing cofilin oligomers in vivo. In the cytoplasm, however, levels of GSSG could rise locally (e.g. in response to oxidative stress), thereby creating an environment that favors the formation of cofilin oligomers in confined areas (see below). However, it is more likely that stabilization of cofilin oligomers in vivo is achieved by other chemical modifications (e.g. phosphorylation) or binding of regulatory proteins.

Cofilin Oligomerization as Dependent on Concentration, pH, and Phosphoinositides—The yield of cross-linked products in the reaction of cofilin with Ellman's reagent decreased with decreasing cofilin concentration. But even at concentrations as low as 2 μM, disulfide-linked dimers and trimers were obtained, suggesting that the Kd value for self-association must be in the low micromolar range. Since physiological concentrations of cofilin are in the range of 5–20 μM (4, 48), it is likely that at least some self-association of cofilin occurs in vivo also, even in the absence of stabilizing agents. In vitro self-association of cofilin was increased at alkaline pH as concluded from our experiments at pH 6.6, where self-association was less pronounced than at pH 8. Moreover, we found evidence that at the lower pH binding to actin prevailed if G- or F-actin was present. This is in line with the observation that bundling of actin filaments by cofilin was more pronounced at pH 8 than at pH 6.6 (38), suggesting a higher degree of cofilin self-association at the higher pH value.

The activity of cofilin has been reported to be regulated by a variety of factors, including binding of phosphoinositides, phosphorylation/dephosphorylation, changes in pH, or binding of regulatory proteins (see Ref. 4 for a review). While looking for a possible implication of these factors in cofilin self-association, we found that PIP2 significantly enhanced oligomerization as measured by the yield of the cross-linking products after treatment with Ellman's reagent. This finding is of interest, because PIP2 also inhibits binding of cofilin to actin (32) as it does with several other actin-binding proteins including profilin (53), gelsolin (54), vinculin (55), and gCap (56). PIP2 binding sites on cofilin have been identified by in vitro studies using peptide mimetics and are located in the long α-helix (α6) and at the N terminus (33, 34). These sites overlap with actin binding sites in the cofilin molecule, indicating that there must be a competition of PIP2 with F-actin for binding to cofilin. Since our data indicate that PIP2 enhances self-association of cofilin, it is tempting to speculate that inactivation of cofilin by PIP2 is accompanied by its oligomerization. If so, self-association of cofilin would be a regulatory event that either prevents actin depolymerizing activity, or switches actin depolymerizing activity to actin bundling activity.

Effect of Cofilin Dimers and Oligomers on Filamentous Actin—Cofilin is known to depolymerize actin filaments in vitro and in vivo (8). In contrast, cross-linked dimers and oligomers lost the ability to depolymerize actin filaments and induced the formation of actin bundles instead, as shown by capillary viscometry, light scattering, and electron microscopy. In electron micrographs, the bundles induced by cofilin oligomers at pH 8 appeared rather straight and sometimes had blunt ends (Fig. 6, A–C). The highly ordered structure suggests that filaments are entirely decorated with cofilin oligomers and that bundling of F-actin occurs in a defined manner. Bundling was also seen with cofilin dimers, but these bundles looked irregular. Moreover, the filaments forming these bundles seemed not to be in tight contact with each other over the whole length of the bundle (Fig. 6D). We suggest that the dimers are unable to decorate actin filaments over their whole length, possibly due to a lower affinity for actin, which in turn may be caused by the lower number of subunits (i.e. the fewer actin binding sites). This argues that bundling activity of cofilin oligomers is due to charge neutralization between the anionic actin filaments and the basic protein cofilin (57). Bundling activity seems not to be restricted to covalently linked oligomers but may be displayed by spontaneously aggregated cofilin molecules as well, as was probably the case in the experiments of McCough et al. (38), who observed bundling activity in electron microscopy experiments in the presence of excess DTT.

The number and size of actin bundles were significantly increased when F-actin solutions were treated not with oligomers only but with a mixture of cofilin oligomers and monomers. At pH 8, large, sheetlike aggregates of actin and cofilin consisting of 50 and more single filaments were formed (Fig. 7A). A possible explanation for this observation is that in this bundling case may include both severing and bundling steps. We imagine that, similar to the continuous process of formation and cleavage of disulfides in a folding protein approaching the correct conformation, severing steps and bundling steps may proceed side by side, until the desired structure is reached. Actin aggregates of similar shape were obtained by others who showed that cofilin or actophorin was able to form bundles from a latticework composed of actin and the actin cross-linking protein α-actinin in vitro (58, 59).

Binding of cofilin and ADF to actin filaments is highly cooperative (22, 36, 37, 38, 52). Cooperativity is explained by a change in the twist of the actin filament, either induced or stabilized by binding of cofilin. This local structural change is thought to facilitate additional cofilin binding at neighboring subunits (24, 38). Since self-association of cofilin was not detected in earlier studies, the change in the filament twist was suggested as the sole explanation for cooperativity. Cofilin self-association, either apart from or in contact with the actin surface, now provides another possibility to explain cooperativity.

Structure of Cofilin Oligomers and Implications for Actin Binding—Both processes, bundling and severing, require binding of cofilin to actin. We anticipated that the two activities of cofilin do not involve actin/cofilin interactions other than those identified so far. In cofilin, two domains, the N terminus and the long α-helix (α6) of human cofilin corresponding to residues 111–128 of human cofilin), are in contact with subdomains 1 and 3 of actin, as shown by cross-linking experiments (60, 61), peptide mimetics (62), mutational analysis (63–65), and molecular modeling (66, 67). Binding to F-actin is believed to require
Cofilin forms oligomers exhibiting actin bundling activity

an additional binding site that interacts with subdomains 1 and 2 of the adjacent (lower) actin subunit, thereby bridging two adjacent actin subunits along the filament (24, 38). This second actin binding site comprises the extended loop at the beginning of β-strand 4 and the C-terminal α-helix (α3 of yeast cofilin, α0 of human cofilin), two domains that are located close together in the three-dimensional structures of ADF and yeast cofilin (65, 67). In agreement with the existence of two actin binding sites in cofilin, mutations in the extended loop or in the C-terminal helix (e.g. by replacement of charged amino acids with glutamine or alanine) impaired binding to F-actin but not to G-actin (24, 63).

The thiols involved in the disulfide links in cofilin oligomers are Cys39 and, most likely, Cys147. Assuming that cofilin oligomers are linear in shape, and considering the position of Cys39 and Cys147 in the tertiary structure of human ADF (65) (taken as a model, since a high resolution structure of human cofilin is not available and human cofilin and ADF are 70% identical in sequence), it is likely that the upper actin binding site, including the N terminus and the long α-helix, is not impaired by disulfide formation in the oligomers. This may be different for the lower binding site, since the second thiol group involved in cofilin oligomerization (most likely Cys147) is located in the C-terminal α-helix, which is part of the second actin binding site. With the second binding site compromised by disulfide formation, binding of cofilin oligomers to actin would only occur through the upper binding site, making F-actin fragmentation impossible. This idea is in line with a recent study that reveals that interaction by both the upper and the lower binding site is necessary to induce filament twist and fragmentation (24). Further support for the importance of the cofilin C terminus for cofilin severing activity comes from the mutagenic studies of a cofilin homologue from C. elegans (68, 69), in which a mutant protein truncated by three residues at the C-terminus showed a loss of F-actin severing activity and weaker F-actin binding than the wild type.

Does Cofilin Play a Role in Actin Filament Bundling Also in Vivo?—All of our experiments discussed so far were done in vitro, but it is tempting to speculate that the bundling activity of cofilin oligomers may be important in vivo also. In cells, the distribution of cofilin correlates well with the established function of the monomer. Distribution of cofilin in quiescent cells is diffuse (10, 14), whereas in motile cells cofilin localizes with actin at the leading edge and in ruffling membranes, where it is thought to enhance the turnover of actin filaments (72). A second localization of cofilin was reported from cells that were submitted to hyperosmotic stress or treated with heat shock or Me2SO. Here, cofilin was additionally found associated with actin in supramolecular structures, so-called actin-cofilin rods, located in the cytoplasm or in nuclei (40). These rods could not be stained with rhodamine phalloidin (40), indicating that the filament structure was altered and/or that the filament surface was saturated with cofilin. Strikingly, these rods appear similar to the structures induced in vitro by cross-linked cofilin oligomers at pH 8 (Fig. 6, B and C).

Besides by hyperosmotic stress, cytoplasmic bundles consisting of actin and cofilin could be induced by overexpression of cofilin (e.g. in yeast (43), in D. discoideum (58), or in mammalian cells (72)). Moreover, actin-cofilin bundles in cytoplasm were induced when exogenous cofilin was microinjected into myogenic cells (48). These findings suggest that actin-cofilin bundles may be formed simply by increasing the concentration of cofilin. An increase in cofilin would also be achieved by dephosphorylation of phosphorylated (inactive) cofilin, an idea that is in line with the finding that the formation of rods in stressed cells is accompanied by dephosphorylation of cofilin (73, 74). Thus, phosphorylation/dephosphorylation of cofilin may regulate both activities of cofilin, severing and bundling, in that exceeding a certain threshold concentration of dephosphorylated cofilin could achieve a shift from severing to bundling activity. Alternatively, such a shift may be induced by binding of regulatory proteins to F-actin-cofilin complexes. A candidate for such a regulatory protein is the recently identified Aip1 (41–43), which interacts with both F-actin and cofilin and enhances the low severing activity of cofilin in vitro.

The function of actin-cofilin rods is unknown. In D. discoideum, cytoplasmic bundles induced by overexpression of cofilin can contract on hyperosmotic shock, suggesting that the bundles might produce force for osmotic shock response and cell migration (58, 70). Interestingly, a recent study revealed a possible implication of actin-cofilin rods in neuronal degeneration (71). Treatment of hippocampal neurons with mediators of neurodegeneration like peroxide or glutamate or depletion of ATP caused the formation of persistent actin-cofilin rods; it was suggested that these are able to disrupt microtubules, thereby causing degeneration of distal neurites and loss of synapses. The latter defect is also described as occurring during Alzheimer’s disease, and it is interesting to note that rodlike actin-cofilin inclusions associated with amyloid plaques were also observed in brains of Alzheimer’s disease patients (71). These findings make it possible that the formation of actin-cofilin rods represents a response to oxidative stress that becomes pathological when the stress persists. Oxidative stress on local areas in the cell may generate peroxides, and their removal consumes GSH under formation of GSGG, which in turn could cause disulfide cross-linking between cofilin subunits as shown in the present study. Such disulfide-stabilized cofilin oligomers, which are potentially pathological, must be distinguished from the noncovalently formed oligomers generated by self-association and their possible physiological function in actin bundling. In line with this distinction, actin-cofilin rods in yeast observed in the absence of Aip1 (42) can only be produced by noncovalent self-association, since yeast cofilin contains only a single cysteine.

In summary, we present evidence that cofilin possesses an intrinsic tendency for self-association and that cofilin oligomers, in contrast to the monomer, are not able to depolymerize actin filaments but induce filament bundling instead. In cells submitted to oxidative stress, cofilin is associated with actin filaments that resemble actin-cofilin structures observed in our in vitro experiments; we therefore conclude that cofilin may exhibit this second, hitherto unknown activity in vivo also.

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