Structure of an F-actin Trimer Disrupted by Gelsolin and Implications for the Mechanism of Severing*

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Stable oligomers of filamentous actin were obtained by cross-linking F-actin with 1,4-N,N'-phenylenedismaleimide and depolymerization with excess segment-1 of gelsolin. Segment-1-bound and cross-linked actin oligomers containing either two or three actin subunits were purified and shown to nucleate actin assembly. Kinetic assembly data from mixtures of monomeric actin and the actin oligomers fit a nucleation model where cross-linked actin dimer or trimer reacts with an actin monomer to produce a competent nucleus for filament assembly. We report the three-dimensional structure of the segment-1-actin hexamer containing three actin subunits, each with a tightly bound ATP. Comparative analysis of this structure with twelve other actin structures provides an atomic level explanation for the preferential binding of ATP by the segment-1-complexed actin. Although the structure of segment-1-bound actin trimer is topologically similar to the helical model of F-actin (1), it has a distorted symmetry compared with that of the helical model. This distortion results from intercalation of segment-1 between actin protomers that increase the rise per subunit and rotate each of the actin subunits relative to their positions in F-actin. We also show that segment-1 of gelsolin is able to sever actin filaments, although the severing activity of segment-1 is significantly lower than full-length gelsolin.

Arguably the most regulated cytoskeletal protein, filamentous actin (F-actin) is critical for cellular motility and mechanical strength, protein sorting and secretion, signal transduction, and cell division. An atomic resolution understanding of the structure of F-actin is a tantalizing goal. The tendency of monomeric actin to form polymers of varying lengths has prevented crystallization and atomic resolution structural analysis of F-actin. To date, all but one of the atomic resolution structures of actin are of the monomer bound to proteins that prevent polymerization, such as DNaseI (2), GS-1 (3), profilin (4, 5), and, most recently, vitamin D-binding protein (6, 7). A structure of uncomplexed monomeric actin labeled with the dye tetramethylrhodamine (8) reveals that actin-binding proteins did not alter the structure of the actin monomer significantly. The structure of an antiparallel actin dimer has been determined (9), but how this structure relates to helical F-actin is unclear.

A model describing F-actin as a helical polymer has been proposed based on fitting the crystal structure of monomeric actin into x-ray fiber diffraction data from oriented actin gels (1). Schutt and co-workers (4, 5, 10) have constructed a topologically different helical model for F-actin by twisting the ribbon-like assembly of actin molecules found in profilin-actin crystals. Transitioning between twisted and untwisted forms of this ribbon was proposed to be a basis for contractile force generation (11–13). To date, the ribbon actin assembly has been observed only in the crystals of profilin-actin heterodimers (4, 5, 10).

A key component to the dynamic assembly and disassembly of actin filaments is the intrinsic hydrolysis of bound ATP. This hydrolysis promotes the dissociation of actin subunits from filament ends. In vivo, actin assembly and disassembly are regulated tightly by binding proteins that likely respond to changes in F-actin structure accompanying ATP hydrolysis (14, 15).

Our goal was to obtain F-actin fragments of defined length for biochemical and high resolution structural analysis. We therefore linked successive actin protomers in F-actin. A specific covalent link is formed between Cys-376 and Lys-193 (in this paper, amino acid residue numbers correspond to chicken skeletal α-actin (16)) of adjacent actin protomers in the filament by N,N'-1,4-phenylenedismaleimide (pPDM)1 (17–19). The ability of purified covalently cross-linked actin oligomers to nucleate actin assembly has been described (20). However, these actin oligomers form filaments under crystallization conditions and are therefore unsuitable for crystallographic analysis.

To block assembly of the purified actin oligomers, we used recombinant segment-1 of gelsolin (GS-1) (21–24) to generate GS-1-bound cross-linked actin oligomers. Because of its strong actin monomer binding activity ($K_d = 5\text{nM}$) (21–24), GS-1 was expected to facilitate depolymerization by both sequestering monomeric G-actin and binding to the fast growing barbed end of newly formed F-actin fragments. Here, we show that mixtures of the cross-linked GS-1-bound actin oligomers can be fractionated readily according to their size and crystallized. We report the crystal structure of the GS-1-bound cross-linked actin trimer to 2.2-Å resolution. Although this structure is

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1 The abbreviation used is: pPDM, N,N'-1,4-phenylenedismaleimide.
Pyrenyl-actin was prepared as outlined (30). Actin was polymerized and several months without any loss in activity. We typically obtained about 3 mM by SDS-PAGE. GS-1 eluted from Affi-Gel Blue was denatured in 6 M Buffer 1. This was then developed with a linear gradient of NaCl to 0.5 M in Buffer 1. GS-1 eluted from this column was stored at –20 °C and Cys-376 of another. The GS-1-complexed actin oligomers were that are cross-linked between Lys-193 and Cys-376 whereas the lower band, however, it is thought that the upper band corresponds to oligomers previously (18, 31, 32). The nature of these two species is not clear; indeed sever actin filaments, although the severing activity of GS-1 alone has no severing activity (3, 27). How-ever, the lack of F-actin severing activity of GS-1 has never been verified using homogeneous samples of recombinant GS-1. Combined structural and biochemical data presented and discussed in this work show that segment-1 of gelsolin does indeed sever actin filaments, although the severing activity of GS-1 is significantly lower than that of full-length gelsolin (28).

MATERIALS AND METHODS

Protein Preparation—GS-1 was purified by from Escherichia coli by overnight growth and expression in the absence of inducing agents employing pGSL1.36 (a generous gift of B. Pope and A. G. Weeds, MRC Laboratory of Molecular Biology, Cambridge). Cells were lysed in Lysis Buffer (4 mM Tris-HCl, pH 8.0, 0.25% sucrose (w/v), 0.4 mM EDTA, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride) by two passes through a French Press at 16,000 p.s.i. The cell lysate was then cycled by centrifugation at 19,500 rpm in a JA-20 rotor (Beckman) for 20 min. The supernatant was passed through a 20-ml DEAE column equili-brated in Buffer 1 (2 mM Tris-HCl, pH 8.0, 0.4 mM EDTA, 0.4 mM CaCl2, 3 mM sodium phosphate, and 5% (v/v) glycerol) and the flow-through was loaded immedi-ately onto a 100-ml Affi-Gel Blue column (Bio-Rad) eluted in Buffer 1. This was then developed with a linear gradient of NaCl to 0.5 M in Buffer 1. GS-1 eluted from this column was –95% pure as judged by SDS-PAGE. GS-1 eluted from Affi-Gel Blue was denatured in 6 M urea and dialyzed overnight into 2 mM Tris-HCl, pH 8.0, 0.5 mM CaCl2. The renatured protein was then separated by Superdex-200 gel filtra-tion chromatography in Buffer 1 containing 20 mM NaCl. This was stored at –80 °C in Buffer 1 with 20 mM NaCl without concentration for several months without any loss in activity. We typically obtained about 100 mg of purified GS-1 per liter of cell culture.

Actin was prepared from chicken muscle as described previously (29). Pyrenyl-actin was prepared as outlined (30). Actin was polymerized and cross-linked with pPDM (Aldrich) at a molar ratio of 1.5 pPDM:actin according to published methods (18) employing a 25 mM solution of pPDM in DMF. Cross-linked F-actin was then harvested by centrifugation at 45,000 rpm for 60 min in a Ti60 rotor (Beckman).

Purified GS-1 in Buffer 1 with 20 mM NaCl was added to the cross-linked F-actin pellet at a 2:1 molar ratio and incubated for 30 min at 25 °C. Typically, we employed 100 mg of cross-linked F-actin and de-natured actin from the Mono-Q 10/10 column (Amersham Biosciences). GS-1 bound oligomers of actin eluted from the Mono-Q according to size elosing a gradient (0.175 to 0.375 M) of NaCl in Buffer 1. To increase the purity of each oligomer, the gradient was held when each oligomer began to elute. The gradient was held until baseline was achieved after the protein had eluted. Oligomers were characterized using SDS-PAGE and mass spectrometry (Voyager-DE™, Perceptive Biosystems). For each oligomer, both an upper and lower cross-linked band is observed with SDS-PAGE, as reported pre-viously (18, 31, 32). The nature of these two species is not clear; however, it is thought that the upper band corresponds to oligomers that are cross-linked between Lys-193 and Cys-376 whereas the lower band may represent cross-linking between Cys-376 of one actin mole-cule and Cys-376 of another. The GS-1-complexed actin oligomers were then concentrated to ~12 mg ml–1 employing filters (Amicon). The GS-1-complexed actin trimer was used fresh for crystallization experiments. Protein concentrations were determined with Bio-Rad protein dye reagent employing G-actin as the standard.

Actin Polymerization and Depolymerization Assays—G-actin (15% pyrenyl-G-actin) was polymerized in 50 mM KCl, 2 mM MgCl2, 1 mM EGTA, and 0.1 mM ATP in 25 mM Tris-HCl, pH 8.0, overnight. To measure the depolymerization of F-actin in the presence of GS-1, various concentrations of GS-1 were added to 2.5 µM F-actin (15% pyrenyl-F-actin), and the fluorescence was monitored in an Amino Bowman Series 2 fluorimeter with an excitation wavelength of 347 nm and emission wavelength of 407 nm. To avoid photobleaching, a 0° polariz-ing filter was used for the excitation beam with a 1-nm slit width. All solutions and reagents were equilibrated to 25 °C before assays were performed. 5 mM CaCl2 was added by removing the cuvette from the fluorimeter, mixing, and reinserting the cuvette into the fluorimeter.

To measure F-actin polymerization in the presence of GS-1-bound cross-linked actin oligomer, 5 µM G-actin (15% pyrenyl-G-actin) was polymerized in 50 mM KCl, 2 mM MgCl2, 1 mM EGTA, and 0.1 mM ATP in 25 mM Tris-HCl, pH 8.0, and the change in pyrene fluorescence was monitored as described above. All solutions and reagents were equilibrated at 25 °C before assays were performed. Varying concentrations of GS-1-bound cross-linked actin oligomer with polymerization salts were added to the G-actin at the beginning of the assay. This mixture was then placed in the fluorimeter. As a result, the start time of the reaction and the start time of data collection are offset slightly.

We fit the following equation to the data obtained from each experiment.

\[
\frac{[F(t) - F_{\text{max}}]}{[F_{\text{max}} - F_{\text{min}}]} = 5 \times \mu M
\]

(Eq. 1)

In the equation, \(F_{\text{max}}\) was the steady-state fluorescence obtained from control actin polymerization reactions employing F-actin as nuclei, \(F_{\text{min}}\) was the minimum fluorescence measured in control reactions of sponta-neous nucleation, and \(F(t)\) was the fluorescence at any time, \(t\), of an experiment.

As discussed in the text, the kinetic and structural data are consistent with actin assembly from the pointed ends of the cross-linked actin oligomers. Therefore, we employed the published polymerization rate constants for ATP-actin at the pointed end of 1 µM−1 s−1 and 0.5 µM−1 s−1 for the \(k_f\) and \(k_r\), respectively (33).

Nucleation Model—The model involves the formation of a nucleus by the binding of an actin monomer to the pointed end of a GS-1-bound cross-linked actin oligomer. In this case, active nuclei, \(N^*\), are in equi-librium with cross-linked actin, \(N\), and G-actin, \(A\), with a forward rate constant, \(k_f\), and a backwards rate constant, \(k_r\).

\[
N + A \rightarrow N^* + A
\]

(Eq. 2)

G-actin then assembles into the nuclei, \(N^*\), with the known \(k_f\), and \(k_r\) for the pointed end, to form the mass of F-actin, \(M\).

\[
N^* + A \rightarrow M
\]

(Eq. 3)

We assume that spontaneous nucleation is insignificant when X-actin is added to the assembly reactions. Because the total concentration of filaments assembled is equal to the total concentration of nuclei formed, assembly of filaments can be described as a function of the concentra-tion of \(N^*\) (34).

We chose to include the contribution of the concentration of nuclei, \(N^*\), into the concentration of actin filaments, \(M\), because the addition of actin monomer to X-actin may contribute to the increase in pyrene signal accompanying F-actin assembly. However, the contribution of \(N^*\) to \(M\) is insignificant in the best-fit simulations, and almost identical curves are generated with and without \(N^*\) in \(M\). We fit the following equations to the experimental data.

\[
d\ln([N] - [N^*]) = -k_f[A][N^*] + k_r[N^*] - k_f[N]dt
\]

(Eq. 4)

\[
d\ln([N] + [N^*]) = k_f[A][N^*] - k_r[N^*] - k_f[N]dt
\]

(Eq. 5)

\[
d\ln(M) = k_f[A][N^*] + k_r[N^*] + k_f[N] - k_r[N]dt
\]

(Eq. 6)
and $2.7 \times 10^{-4}$ $\mu M$ $s^{-1} \pm 2.6\%$ (S.E.; $n = 8$) for the GS-1-bound cross-linked actin dimer and trimer, respectively. The average best-fit $k_b$ values for the dimer and trimer were 0.026 $s^{-1} \pm 9.0\%$ (S.E.; $n = 7$) and 0.024 $s^{-1} \pm 3.7\%$ (S.E.; $n = 8$), respectively. The root mean square deviations ranged between 0.0118 and 0.0320 for the calculated curves fitting the GS-1-bound cross-linked actin dimer nucleated data set and 0.0126 and 0.0426 for the trimer curves. An additional round of simulation was performed employing the average $k_b$ and $k_t$ values from the initial simulations and fitting the start time, $t_0$, and initial G-actin concentration to obtain the curves shown in Fig. 2. Initial G-actin concentration was fit to account for possible pipetting errors that can influence the steady-state pyrene fluorescence measured. The average best-fit starting G-actin concentration for the calculated dimer-nucleated assembly curves was 5.3 $\mu M \pm 1.09\%$ (S.E.; $n = 7$) and 5.2 $\mu M \pm 1.47\%$ (S.E.; $n = 8$) for the trimer curves.

**Crystallization of GS-1-complexed Actin Trimer—** A solution at $-12$ mg $mL^{-1}$ of the GS-1-complexed actin trimer was used for crystallization by the vapor diffusion method. For crystals of the complex that were grown in the presence of ATP, 2 $\mu L$ of protein solution were mixed with 2 $\mu L$ of reservoir buffer containing 15% (w/v) polyethylene glycol 1000, 25 mM Tris-HCl, pH 8.0, 2 mM ATP, 100 mM MgCl$_2$, 2 mM CaCl$_2$, and equilibrated against this buffer for 5–7 days at 4°C. Crystals of the GS-1-complexed actin trimer in the presence of ADP were grown under the same conditions using 8% (w/v) polyethylene glycol 3350, 25 mM Tris-HCl, pH 8.0, 2 mM ADP, 100 mM CaCl$_2$, and 2 mM MgCl$_2$ as reservoir buffer. Before data collection, both crystal forms were cryo-protected by adding glycerol to a final concentration of 15% and then flash-frozen in liquid nitrogen.

**Identification of Molecules in Crystals—** To confirm the presence of GS-1-bound actin trimers in crystals, crystals grown in the presence of either ATP or ADP were harvested, washed, and analyzed using SDS-PAGE and mass spectrometry (Voyager-DECTM, PerSeptive Biosystems). Results of SDS-PAGE and mass spectrometry analysis confirmed the presence of GS-1 and actin trimer in both crystal forms. Neither actin dimers nor monomers were detected in the crystals.

**Data Collection, Model Building, and Refinement—** For both crystal forms, x-ray diffraction data were measured at $-190$ °C. The data in both cases were collected to 2.2 Å at Advanced Light Source (Lawrence Berkeley National Laboratory) beamline 5.0.2 (A = 1.1 Å). Both data sets were integrated using DENOZo and scaled with SCALEPACK (35). Both crystal forms were of the monoclinic space group $P2_1$ with two GS-1-complexed actin molecules in the asymmetric unit and cell dimensions of $a = 67.2 \AA$, $b = 75.9 \AA$, $c = 96.8 \AA$, $\alpha = 91.9\%$ and $a = 66.7 \AA$, $b = 75.9 \AA$, $c = 84.9 \AA$, $\beta = 90.1\%$ for the crystals grown in the presence of ADP and ATP, respectively.

Both structures were determined by the molecular replacement method (CNS; see Ref. 36) using atomic coordinates for the GS-1-complexed rabbit actin (3). Electron density maps based on coefficients up to $F' - F$ were calculated from the phases of the initial models. Subsequent rounds of model building and refinement were performed using programs QUANTA (Molecular Simulations, Inc.) and CNS (36), respectively. Electron density corresponding to the bound nucleotide was well defined in all maps calculated for both structures. In both cases, ATP was bound in the nucleotide pocket. Both structures were virtually identical. However, because both the quality of x-ray data and the current refinement statistics are better for the structure obtained from the crystals grown in the presence of ADP, the latter structure is described in this paper. The current structure is refined to $R_{rms}$ values of 19.3/23.3 using all data in the resolution range of 25.0 to 2.2 Å ($F > 0$, a bulk solvent correction was applied; see Table I). Both GS-1-complexed actin protomers present in the crystal asymmetric unit include two GS-1-bound ATP and two additional Ca$^{2+}$-ions. Residues 2–125 and 3–125 are built for two GS-1 segments, and residues 7–41, 50–375 and 7–44, 51–374 are built for two actin molecules, respectively. There are 471 water molecules in the asymmetric unit of the current model.

**F-actin Severing Assay—** $5 \mu M$ F-actin (15% pyrenyl-actin) was incubated with 1.5 $\mu M$ GS-1 in 50 mM HCl, 2 mM MgCl$_2$, 1 mM EGTA, and 0.1 mM CaCl$_2$. In 25 mM Tris-HCl, pH 5.0, in 100 mM total in the presence or absence of 5 mM CaCl$_2$ for 10 min at 25°C. 20 $\mu L$ of stock 20 $\mu M$ G-actin (15% pyrenyl-actin) in 10 mM Tris-HCl, pH 8.0, 0.3 mM CaCl$_2$, 0.1 mM EDTA, 0.2 mM $\beta$-mercaptoethanol, and 0.2 mM ATP was then added to the reaction (final concentration $3.3 \mu M$ G-actin), and the change in fluorescence was monitored. The initial rate of elongation for each concentration was employed to determine the concentration of free pointed ends in the assay, assuming that GS-1 remains bound to the barbed ends of filaments, employing the known on-rate for ATP-actin at the pointed end of 1 $\mu M^{-1} s^{-1}$ and the relationship $d[F/dt = k_r[ends][G-actin]$. 

**RESULTS**

**Purification of GS-1-bound Actin Oligomers—** We polymerized F-actin purified from chicken and treated it with pPDM, which cross-links specifically between Cys-376 and Lys-193 of two adjacent actin protomers in a filament (17–19). This F-actin was then depolymerized in the presence of excess recombinant GS-1 to generate GS-1-bound cross-linked actin oligomers of varying lengths (see “Materials and Methods”). The heterogeneous mixture of GS-1-bound actin oligomers was resolved by a combination of gel filtration and anion exchange chromatography to yield purified GS-1-bound cross-linked actin oligomers of defined length (Fig. 1). The identification of purified cross-linked actin oligomers was confirmed by mass spectrometry (data not shown).

**Cross-linked Actin Oligomers Nucleate Actin Polymerization—** Both GS-1-bound cross-linked actin dimers and trimers nucleated actin assembly (Fig. 2, B and C), whereas GS-1-bound actin monomer purified from the same cross-linking reaction inhibited even spontaneous nucleation (Fig. 2A). No free GS-1 was detected in these polymerization reactions (data not shown), suggesting that GS-1 remains bound to each subunit of the purified actin oligomer during the assembly reaction.

The average steady-state concentration of F-actin formed when GS-1-bound cross-linked actin oligomers were employed as nuclei was only 4.4 $\mu M \pm 0.77\%$ (±S.E.; n = 11). Given that 5 $\mu M$ G-actin was included in these actin assembly reactions, this F-actin steady-state concentration is consistent with pointed end growth, where the critical concentration is known to be about 0.6 $\mu M$ (33).

The concentration of GS-1-bound cross-linked actin oligomers required for a substantial increase in assembly rate is in the micromolar range, whereas nanomolar concentrations of pPDM cross-linked actin trimer purified with no GS-1-bound nucleates rapid assembly (20). The presence of a lag phase at the beginning of elongation assays with added GS-1-bound cross-linked actin oligomers indicates that the rate of nucleation is slower than the rate of elongation. This suggests that the added GS-1-bound cross-linked actin oligomer undergoes a transition from a nucleation-incompetent form to a nucleation-competent form. This transition may occur through a conformational change in the actin oligomer itself or when the first actin monomer binds to the pointed end of the cross-linked actin oligomer. Modeling of the assembly data (see “Materials and Methods,” and the calculated curves in Fig. 2, B and C) are consistent with the latter.

The average on-rates, $k_1$, for the first actin monomer binding to either the cross-linked actin dimer or the trimer are similar.
Fig. 2. Polymerization of actin in the presence of varying concentrations of X-actin oligomers. 5 μM G-actin (15% pyrenyl-G-actin) was polymerized as described under "Materials and Methods" with varying concentrations of purified X-actin oligomers. Raw fluorescence measurements were converted to μM F-actin according to the increase in pyrene fluorescence over time. A, addition of 2 μM F-actin seeds (red) results in rapid elongation without an initial lag phase, whereas spontaneous nucleation (blue) results in very slow polymerization. The addition of GS-1-bound actin monomer at various concentrations (2 μM, black; 1 μM, green; and 0.1 μM, pink) inhibits even spontaneous nucleation. B, addition of GS-1-bound cross-linked actin dimer results in an increased rate of polymerization dependent on the concentration of dimer added (1.5 μM, red; 1.0 μM, blue; 0.5 μM, green; 0.35 μM, black; 0.25 μM, pink; 0.15 μM, cyan; 0.1 μM, yellow). Plotting symbols show 16.6% of the data points for clarity. Drawn curves show the best fit to the experimental data employing the model and the average kinetic constants outlined under "Materials and Methods." C, addition of GS-1-bound cross-linked actin trimer results in an increased rate of polymerization dependent on the concentration of trimer added (1.0 μM, red; 0.75 μM, blue; 0.5 μM, green; 0.35 μM, black; 0.25 μM, pink; 0.2 μM, cyan; 0.15 μM, yellow; 0.1 μM, orange). Plotting symbols show 16.6% of the data points for clarity. Drawn curves show the best fit to the experimental data employing the model and the average kinetic constants outlined under "Materials and Methods."
extended filamentous actin structure emerges (Fig. 5B). This filament, although untwisted and more extended than F-actin (rotational symmetries are 180 and 167 degrees), and axial translations per subunit are 75.9 and 55.0 Å in crystalline GS-1-bound actin trimer and F-actin, respectively; see Fig. 5, B and C), possesses the general topological hallmarks of the helical model of F-actin (1). Specifically, subdomain 2 and the N terminus (Fig. 5, B and C, red and yellow, respectively) in each actin subunit are positioned at higher radius whereas subdomain 3 (Fig. 5, B and C, blue) is located at lower radius in both actin filaments. The hydrophobic loop (Fig. 5, B and C, cyan) is positioned in the center in both cases. Because of the topological similarity between the helical model of F-actin and the filament observed in the GS-1-complexed actin trimer crystal, we term the GS-1-complexed actin trimer structure an “extended” F-actin (X-actin; see Fig. 5B). The observed distorted symmetry of X-actin compared with that of F-actin appears to result from the binding of GS-1.

Fig. 4. Structure of the GS-1-complexed actin trimer. Actin subunits in the trimer complex are colored in dark green, orange, and blue. The corresponding segments of gelsolin are light green, yellow, and cyan. The structure of each GS-1-bound protomer in the trimer is similar to the structures of GS-1-complexed actin solved previously. In each protomer, the bound nucleotide, Ca\(^{2+}\)-ATP, is shown as a gray space-filling model. Coordinated ions of Ca\(^{2+}\) are drawn as red spheres. The pPDM cross-link is between Lys-193 of one actin subunit and Cys-376 of its neighbor. In each actin subunit, the C-terminal residues 375–377 and residues corresponding to DNase I binding loop are disordered and are not included in the model. The dashed lines, therefore, connect the cross-linked Lys-193 of subunits 1 and 2 to the last visible C-terminal residue, Arg-374, of subunits 2 and 3, respectively.

Fig. 3. Stereo view of electron density corresponding to the bound Ca\(^{2+}\)-ATP in GS-1-bound actin trimer crystallized in the presence of ADP. Simulated annealing composite omit map was calculated for the refined model and is displayed at 1.0 σ in gray. The bound ATP and Ca\(^{2+}\) are shown as a stick model and yellow sphere, respectively. Molecules of water coordinating the bound cation are shown as red spheres.
GS-1 Severs Actin Filaments in the Presence of Ca$^{2+}$—The observed stoichiometry and symmetry of the GS-1-bound actin trimer suggested that segment-1 of gelsolin might intercalate between actin protomers in F-actin and possibly sever actin filaments. Additional experiments (Fig. 8) aimed to verify this hypothesis showed that the rate of F-actin depolymerization in the presence of Ca$^{2+}$ and excess GS-1 varies with different GS-1 concentrations (Fig. 8A). These data argue against simple monomer sequestration as the mechanism of GS-1-mediated F-actin depolymerization, which would rely solely on the off-rate of actin monomers from F-actin. Furthermore, the concentration of free pointed ends in F-actin that were incubated with a substoichiometric concentration of GS-1 was calculated employing the initial rate of polymerization after additional G-actin was added to the reaction (Fig. 8B). This concentration is higher in F-actin-GS-1 reactions in the presence of Ca$^{2+}$ (3.4 nM) than that in the absence of Ca$^{2+}$ (0.6 nM). The latter data suggest the production of new ends as a result binding of GS-1 and support the hypothesis that GS-1 is able to sever F-actin.

**DISCUSSION**

Combined results of our structural and biochemical studies clarify several important aspects of actin dynamics and its regulation.

**Segment-1 of Gelsolin Severs F-actin—**Actin assembly is tightly regulated by specialized actin binding proteins. Among these is gelsolin, an 84-kDa protein that remodels the actin cytoskeleton during cell movement, apoptosis, and cytokinesis (reviewed in Refs. 23, 24, and 28). To accomplish this, gelsolin binds, severs, and caps actin filaments in a Ca$^{2+}$-regulated manner (23, 24, 28, 39).

Gelsolin consists of six segments that share significant sequence similarity (38). Numerous studies have focused on attributing the activities of gelsolin to its segments (3, 21, 39, 42, 43). Initially, proteolytic fragments of gelsolin were tested. The first work with chymotryptic segment-1 of gelsolin (GS-1) suggested it might sever actin filaments (25). However, subsequent work suggested that chymotryptic GS-1 preparations were contaminated with a small amount of a longer fragment of gelsolin (GS-1–3), which was responsible for the observed severing activity (26). Therefore, it is now commonly held that GS-1 alone has no severing activity (3, 27).
Recombinant techniques permit purification of homogeneous preparations of specific segments of gelsolin where contamination with full-length gelsolin is not possible. Because the actin monomer binding activity of recombinant GS-1 has been studied extensively (3, 22), in our experiments on preparation of F-actin fragments (Fig. 1), recombinant GS-1 was expected to facilitate depolymerization by both sequestering monomeric G-actin and binding to the fast growing barbed end of newly formed F-actin fragments.

However, atomic resolution structural analysis of GS-1-bound actin trimers (Fig. 4) suggested that, in the presence of Ca2+, GS-1 intercalates between actin subunits in the filament, perturbing both the arrangement of the protomers in F-actin (Fig. 5) and the intersubunit contacts responsible for stability of the actin filament (Fig. 6). Consistent with the structural data, results of our biochemical experiments (Fig. 8) showed that GS-1 mediates depolymerization of F-actin in a Ca2+-dependent manner by severing the actin filaments. The different rates of depolymerization of F-actin at different excess concentrations of GS-1 argue against a simple monomer sequestration model, which would rely solely on the off-rate of actin monomers from F-actin, and suggests that GS-1 plays a severing role in the depolymerization of F-actin. Our biochemical data also shows that GS-1 produces new actin filament pointed ends for nucleation, again consistent with a severing mechanism.

The concentration of free pointed ends (3.4 nM) in the presence of Ca2+ was much less than the concentration of GS-1 employed (1.5 μM). This is consistent with a very inefficient severing protein, compared with full-length gelsolin, which produces one break for every molecule (28). The observed difference could be explained by the absence of segments-2–3, which may play the role of binding to the side of F-actin and correctly position GS-1 on the F-actin surface (3, 27, 39, 42). Without GS-2–3 segments, correct positioning of the GS-1 along the actin filament for severing may rely on random interactions with the filament and may therefore be inefficient.

GS-1-bound Cross-linked Actin Oligomers Retain Important Structural Features of F-actin and Nucleate Actin Polymerization—GS-1 present at a 2:1 ratio depolymerizes cross-linked F-actin completely, resulting in a mixture of GS-1-bound cross-linked actin oligomers of varying lengths than can be separated according to their size (Fig. 1) and analyzed. Crystallographic analysis of GS-1-bound actin trimer showed that, although perturbed by the binding of GS-1, it retained the general topological features of helical F-actin (1) (see Figs. 4 and 5).

Modeling of GS-1-mediated actin filament assembly data (Fig. 2C) suggests that the cross-linked actin trimer undergoes a conformational change when the first actin monomer binds to its pointed end, providing a proper interface for an incoming actin monomer to bind, thereby forming a nucleus for assembly. These data indicate that, although arranged by the 21 symmetry in the crystal (Fig. 4), the protomers of the actin trimer may be flexible in solution. Retaining their close association, the protomers within the actin trimer in solution are most likely free to rotate and adopt a structure that is able to bind an actin monomer and form an actin nucleus.

Of interest is the finding that low concentrations of GS-1-bound actin monomer suppress spontaneous F-actin assembly (Fig. 2A). GS-1-bound actin monomer most likely binds the fast growing barbed end of rare actin dimers or trimers formed spontaneously, thereby inhibiting their potential to nucleate polymerization (44). Because the concentration of dimers and trimers formed spontaneously is very low compared with free monomer, added GS-1-bound actin dimer or trimer is far more likely to interact with monomer. According to the best-fit kinetic model, these more frequent interactions of GS-1-bound cross-linked actin oligomers with free actin monomer lead to nucleation.

Rotational Freedom of F-actin Is Important Functionally—Cell biology experiments have demonstrated beautifully the
dynamic features of actin fibers (reviewed in Ref. 45). An emerging theme of F-actin structure is the importance of rotational freedom of actin protomers in the filament. The ability of F-actin to tolerate such rotational freedom is demonstrated most dramatically by the structure of cofilin-decorated F-actin, where the filament is super-twisted by 5 degrees and still exists as a polymer (46). Changing the twist of F-actin is most likely a major component to the severing activity of many proteins including gelsolin. Recently, structural analysis of F-actin has shown that it can exist as a super-twisted polymer in the absence of other proteins (47, 48). This mode of F-actin is thought to involve a change in the way that the subunits of F-actin interact with each other (48).

Rather than being super-twisted, our X-actin structure is under-twisted by 13° relative to normal F-actin because of the GS-1 binding (see Figs. 4 and 5). With GS-1 removed, the X-actin filament can be transformed into the helical model of F-actin by combination of translational (−10.4 Å) and rotational (13° twist with additional tilt) movements of each of the actin protomers within the filament. These movements would result in the filament twisting and shortening (an animation of this transition is available as Supplemental Material). Interestingly, like the super-twisted polymer observed by Egelman and co-workers (48), under-twisted X-actin employs an alternative arrangement of the interacting elements compared with that found at the interface in F-actin (Fig. 6). The ability of the F-actin interface to accommodate rotational movements of the subunits within the filament and to adjust radically in different actin self-associations may be inherent in the evolved design of the actin surface. Furthermore, this property of actin might provide a structural basis for the variable twist of the F-actin helix observed experimentally (49, 50) and could be important for the dynamics of the actin filament. It should be noted that, in contrast to extensive data on variable twist within F-actin, there are no observations of significant extension of F-actin structure along its axis. It is therefore possible that the described extended actin trimer structure caused by the intercalation of GS-1 might exist only within crystals.

Rotational freedom of the actin subunits also explains a paradoxical structural feature of the X-actin trimer (Fig. 4). Because of the non-equivalent nature of the cross-linked residues, we anticipated all three actin protomers of the complex bound covalently to be in the same crystal asymmetric unit. Surprisingly, we found each GS-1-bound actin protomer in a different asymmetric unit with the first and the third protomers being in neighboring unit cells. This apparent paradox was resolved by the realization that the covalent linkages tethering the protomers in the trimer complex are flexible. This flexibility results in both pPDM and the last three C-terminal residues of each actin protomer (including Cys-376 to which the cross-linker is attached) being disordered and not visible in our electron density maps. This flexibility also explains the fact that the assembly of the three actin protomers in the complex shows a strict rotational and translational symmetry corresponding to a crystalllographic 2-fold screw axis (Fig. 4). As we already discussed, although arranged by the $2_1$ symmetry in the crystal lattice, the protomers of the actin trimer most likely are free to rotate in solution.

Structural Transitions between Helical F-actin- and Profilin-Actin-like Ribbons Are Not Possible—Our analysis of the crystal lattice revealed an additional arrangement of the GS-1-bound actin subunits. This arrangement (Fig. 7B, blue), which is found at the interface between two strands of actin trimers coming from different X-actin oligomers, is strikingly similar to that previously found exclusively in profilin-actin crystals and called the ribbon structure (4, 5). The distance between Lys-193 (Fig. 7B, green) of one actin subunit and the C terminus (Fig. 7B, red) of the next subunit in the ribbon between X-actin trimers approaches 40 Å. This is too far to be cross-linked by pPDM, which spans 11.4 Å (37). In contrast, the distance between Lys-193 and the last visible C-terminal residue of the neighboring subunits in the X-actin arrangement is about 18 Å (Fig. 7A, green and red, respectively). This arrangement accommodates the covalent cross-link between the Lys-193 and Cys-376, given some flexibility of the C-terminal tail. Our analysis shows that the X-actin trimer is derived from a cross-linked helical filament whereas the profilin-actin-like ribbon arrangement cannot result from untwisting a single F-actin filament. Like other experimental data, the actin assemblies found in the GS-1-bound actin trimer crystals rule out the models both for F-actin and for muscle contraction based on a single F-actin filament transitioning between the helical and ribbon forms described by Schutt et al. (11–13).

Nucleotide and Actin Dynamics—It is intriguing to note that Ca$^{2+}$-ATP was found in the nucleotide binding pocket of the X-actin trimer structure (Fig. 3) even though the trimer was created from F-actin that presumably contained ADP, and the crystals were formed in the presence of ADP (see “Materials and Methods”). It would appear that the ATP present in solution at the time that the GS-1 was used to disassemble the actin filaments exchanged for ADP in the nucleotide pocket and then remained there bound tightly. Although the higher affinity of uncomplexed monomeric actin for ATP is well documented ($K_a$ for ATP is 70 pM and for ADP is 12 nM (33)), the absence of the nucleotide exchange in the case of GS-1-bound...
actin trimer was not expected. In the presence of 1 mM ADP and in the absence of any ATP in crystallization mother liquor, the reported off-rate of ATP for G-actin (0.015 s\(^{-1}\)) (33) would allow the bound ATP to exchange for ADP. Under similar conditions, two structures of monomeric actin have been crystallized with ADP bound in the active site (2, 7). In the case of GS-1-bound actin trimer, however, electron density maps reveal that highly ordered ATP occupies the nucleotide pocket of actin subunits fully (Fig. 3). This result suggests that the binding of GS-1 limits the exchange of nucleotide in the complex. Tight binding of ATP in actin-gelsolin complexes has been reported previously (51). The following structural analysis offers an explanation for the preferential binding of ATP by the GS-1-complexed actin.

A superposition of the actin structure from the GS-1-complexed actin trimer with available actin structures revealed that all of them, although differing in details, form two distinct categories. The first group, which includes the structure of actin from the GS-1-complexed actin trimer (as well as all structures of actin complexed with GS-1 reported previously; thin gray lines in Fig. 9A), can be defined as the “closed” category (Fig. 9A, red and pink) as it contains the closed structure of actin found in one actin profilin complex (4). The second category includes the “open” conformation of actin from a different actin profilin complex (5) and is, therefore, defined as open (Fig. 9A, cyan). Detailed structural analysis of the closed and open structures of actin suggested at the major difference between these two conformations, closing or opening of the nucleotide binding cleft, could be explained by combination of two rotational movements (52). The first rotation would involve semi-rigid subdomain 2 moving around a hinge at the base of subdomain 2. The second rotation would involve two domains of actin (subdomains 1 plus 2 and 3 plus 4, respectively) moving relative to each other around the extended hinge (or shear, amino acid residues 137–152; see Fig. 9A, yellow and orange) area of actin (52). Comparative structural analysis, performed in this and related work (15) on twelve actin structures (Fig. 9A), supports this proposal. This analysis, as well as crystallographic study of actin-related proteins Arp2/3 (53), suggest that the closed and open structures of actin could be relevant physiologically and might represent, respectively, the ATP/ADP and inorganic phosphate and ADP/nucleotide free-like conformational states of actin (15).

Based on this analysis, we propose that the binding of GS-1 stabilizes the closed ATP-like conformation of actin. We also suggest a mechanism for the preferential binding of ATP by the GS-1-complexed actin. Fig. 9A shows that GS-1 intercalates between subdomains 1 and 3 in the sheared area, requiring the two actin domains to rotate with respect to each other (Fig. 9A, black arrows) and to adopt the closed conformation as GS-1 cannot fit into the open structure. This rotational movement brings the nucleotide binding loops of actin (Gln-14–Lys-20 and Asp-156–Gly-160; see Fig. 9B, red) closer in space. By forming hydrogen bonds with two conserved residues (Ser-16 and Gly-160), the \(\gamma\)-phosphate of ATP bridges the nucleotide binding loops stabilizing their closed conformation. Formation of an extended related network of side- and main-chain interactions (Fig. 9B) stabilizes the closed conformation of actin further, making strong binding of ATP by the GS-1-complex complex favorable. Locked rigidly in the closed conformation, the ATP-bound GS-1-complexed actin likely has a limited possibility of “breathing,” which would be required for nucleotide exchange to occur.

Implications for the Mechanism of Severing by Gelsolin—The observed preferential binding of ATP and the absence of nucleotide exchange in actin complexed with GS-1 might be relevant functionally and could constitute a part of the gelsolin mechanism (23, 24, 28, 39). Barbed ends containing ADP-bound actin subunits grow more slowly than barbed ends containing ATP-bound actin (33). The proposed gelsolin mechanism could involve recreating an ATP cap at the barbed ends of severed F-actin fragments. After dissociation of gelsolin, the newly formed F-actin fragments with ATP-bound actin subunits at the barbed ends would regain their ability for the fast growth.

According to the most recent models of gelsolin action (3, 39) (reviewed in Refs. 23, 24, and 28), segments-2 and -3 of gelsolin bind to the side of an actin filament first. Bound to F-actin, these segments position GS-1 to intercalate between actin subunits in the filament in a calcium-dependent manner. The severing model of McLaughlin et al. (3) suggests that the binding of GS-1 to a subunit of F-actin produces steric clashes between the GS-1-bound barbed end of one subunit and the pointed end of the adjacent subunit. It was predicted that only these longitudinal contacts are affected directly by the intercalation of GS-1 but that enough of the stronger bonds along the actin strand are broken to fragment the filament (3).

The crystal structure of GS-1-bound cross-linked actin trimer in the X-actin arrangement provides the first direct picture of the result of gelsolin severing activity on F-actin. In support of the model proposed by McLaughlin et al. (3), we find that the longitudinal actin-actin contacts along the filament are broken to accommodate GS-1 (Fig. 6A). As a result, across-strand bonds are also distorted (Fig. 6C), and the actin protomers are able to rotate with respect to each other along the helix axis. These distortions allow the normal actin filament helical structure to be relaxed. With the longitudinal contacts broken, the distorted across-strand bonds are not strong enough to maintain the helical integrity of the filament, and it breaks at the point of the GS-1 insertion. Our structure suggests that these atomic-scale distortions translate into an untwisting and eventual breakage of the actin helix where the GS-1 is bound. An animation (available as Supplemental Material) showing the hypothetical transition between F-actin fragment and GS-1-bound actin oligomer is made to show the relationship between actin subunits before and after the GS-1 binding.

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