Actin: From Cell Biology to Atomic Detail

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Received September 11, 1995, and in revised form March 14, 1997

Over the past 2 decades our knowledge about actin filaments has evolved from a rigid “pearls on a string” model to that of a complex, highly dynamic protein polymer which can now be analyzed at atomic detail. To achieve this, exploring actin’s oligomerization, polymerization, polymorphism, and dynamic behavior has been crucial to understanding in detail how this abundant and ubiquitous protein can fulfill its various functions within living cells. In this review, a correlative view of a number of distinct aspects of actin is presented, and the functional implications of recent structural, biochemical, and mechanical data are critically evaluated. Rational analysis of these various experimental data is achieved using an integrated structural approach which combines intermediate-resolution electron microscopy-based 3-D reconstructions of entire actin filaments with atomic resolution X-ray data of monomeric and polymeric actin.

INTRODUCTION

The molecular building blocks of the eukaryotic cytoskeleton include actin, intermediate filament proteins, and tubulin together with their respective associated proteins. Actin is a highly conserved eukaryotic protein that is expressed in most animals, plants, and fungi in multiple, tissue-specific, and developmentally regulated isoforms (for review, see Hennessy et al., 1993; Sheterline et al., 1995). In vivo, roughly equal amounts of actin are unpolymerized (termed G-actin) and in filamentous form (termed F-actin). In vitro, actin is monomeric under low ionic strength conditions, e.g., 0.2 mM Ca\(^{2+}\) or 0.05 mM Mg\(^{2+}\). Elevating the ionic strength to physiological conditions (e.g., 2 mM MgCl\(_2\)/100 mM KCl) causes monomeric G-actin to polymerize within minutes into synthetic F-actin filaments. Figure 1a illustrates that a macroscopic indication of actin polymerization is a sharp rise in the viscosity of the solution: G-actin solutions have a viscosity that is close to that of water (top cuvette), whereas F-actin is very viscous, as evidenced by the trapped bubbles (bottom cuvette). Judged by light and electron microscopy, synthetic F-actin filaments can become many micrometers long (see Borejdo and Burlacu, 1991; Burlacu et al., 1992). Micrographs of such synthetic F-actin filaments are shown after different preparation and electron microscopic imaging: Fig. 1b reveals a negatively stained specimen imaged at high magnification in a scanning transmission electron microscope (STEM) by the annular dark-field (ADF) mode; Fig. 1c displays a completely unstained speci-

\(^{3}\) Abbreviations used: G-actin, monomeric actin; F-actin, synthetic actin filaments; EM, electron microscope/microscopy; CTEM, conventional transmission electron microscope/microscopy; STEM, scanning transmission electron microscope/microscopy; ADF, annular dark-field; BF, bright-field; FESEM, field-emission scanning electron microscope/microscopy; 2-D, 3-D, two-, three-dimensional; S-1, myosin subfragment-1; HAS, high-affinity divalent cation binding site.
Fig. 1. Actin polymerization, actin filaments, and actin-based motility. (a) (Top) A quartz cuvette containing G-actin at a protein concentration of 1 mg/ml. (Bottom) The same cuvette 15 min after adding MgCl₂ to 2 mM and KCl to 50 mM. Small air bubbles are formed during polymerization of G- into F-actin. The larger bubbles have been introduced artificially to demonstrate that the viscosity of F-actin is significantly higher than that of the monomeric G-actin solution. (b–d) Different preparations of F-actin filaments: (b) negatively stained with 0.75% uranyl formate and imaged in a scanning transmission electron microscope (STEM) by the annular dark-field (ADF) mode; (c) frozen-hydrated and phase-contrast imaged in a conventional transmission electron microscope (CTEM) (courtesy of Dr. Ron Milligan, Scripps Research Institute, La Jolla, CA); and (d) freeze-dried/rotary metal-shadowed and imaged by secondary electrons in a field-emission scanning electron microscope (FESEM) (courtesy of Dr. Roger Wepf, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany). (e) Actin-based invitro motility assay. The experimental setup for an in vitro motility assay is relatively simple. A flow cell, i.e., an open system that allows the exchange of solutions during the experiment, is assembled from a glass slide and a nitrocellulose-coated coverslip using two parallel lines of silicon grease. A myosin solution is then allowed to flow into the cell. Within seconds, the myosin molecules bind to the coverslip. After a washing step to remove excess myosin, actin filaments that have been labeled with fluorescent phalloidin (TRITC-phalloidin; courtesy of Dr. H. Faulstich, Max-Planck Institute for Medical Research, Heidelberg, Germany) are added to the flow cell. These fluorescent actin filaments bind to the myosin molecules and the motility assay system is now ready for use. Adding ATP results in a sliding movement of actin filaments. Three video frames (i.e., after t = 0, 4, and 8 sec of a particular area of interest) have been recorded after addition of ATP. Three filaments have been marked with arrowheads in each frame to follow their myosin-driven movement. Scale bar, 25 nm (b–d).
men preserved in a near-native state in a thin film of amorphous ice and phase-contrast imaged in a conventional transmission electron microscope (TEM); and Fig. 1d yields a freeze-dried/rotary metal-shadowed sample imaged by secondary electrons (SE) in a field-emission scanning electron microscope (FSEM).

To reconstitute molecular force generation in a minimal test tube system, elegant in vitro motility assays have been developed that require a molecular motor (e.g., myosin), a molecular “track” (e.g., synthetic F-actin filaments), and fuel (i.e., ATP) (for details, see Kron et al., 1991). These systems have significantly advanced our current view of the biochemistry and biophysics of the mechanochemical ATPase cycle (Finer et al., 1994), the functional anatomy of the myosin molecule (Lowey et al., 1993a,b), and the role of the actin filament in generating directed force during muscle contraction or cellular motility (Prochniewicz and Yanagida, 1990; Schwyter et al., 1990). Light microscopy allows imaging of dynamic biological processes in their native aqueous environment with relatively high temporal resolution (i.e., subsecond regime). As an example, in Fig. 1e a time-lapse video sequence of unidirectionally moving F-actin filaments driven by myosin subfragment-1 (S-1) immobilized on a nitrocellulose-coated coverslip is presented. For visualization, the actin filaments were labeled with fluorescent phalloidin (TRITC-phalloidin) and imaged by a video-enhanced low-light-level camera in an epifluorescence microscope.

Because conditions favoring crystallization of G-actin also induce its polymerization into F-actin filaments, it has been difficult to obtain 3-D crystals of pure actin suitable for X-ray diffraction analysis. To overcome this problem, several investigators have complexed actin with proteins that inhibit its polymerization and thereby obtained cocrystals. As a result of these efforts, the atomic structures of actin in complex with DNase I (Kabsch et al., 1990), gelsolin segment 1 (McLaughlin et al., 1993), and profilin (Schutt et al., 1993) have now been determined. Comparison of the different actin structures has revealed that whereas their overall structures are remarkably similar, they exhibit a number of significant local differences which may be due in part to the different proteins with which they were complexed, but also to the fact that different isoforms were investigated (i.e., skeletal muscle a-actin in the case of the actin–DNase I and the actin–gelsolin segment 1 cocrystals, and bovine p-actin in the case of the actin–profilin cocrystals). Based on the atomic structure of skeletal muscle actin determined by Kabsch et al. (1990), atomic models for synthetic F-actin filaments (Holmes et al., 1990; Lorenz et al., 1993), muscle thin filaments (Lorenz et al., 1995), and S-1-decorated actin filaments (Rayment et al., 1993; Schroeder et al., 1993) have been proposed, and these have been compared with EM-based 3-D helical reconstructions of corresponding specimens (e.g., Bremer and Aebi, 1992; Rayment et al., 1993; Schroeder et al., 1993; Bremer et al., 1994; Egelman and Orlova, 1995a; Hoenger and Aebi, 1996). A number of studies have shown that actin filaments are intrinsically dynamic (Aebi et al., 1986; Carlier, 1991; Bremer et al., 1991, 1994; Steinmetz et al., 1997) and that this dynamics can be modulated by effector molecules such as phalloidin or the state of hydrolysis of the bound nucleotide (Janmey et al., 1990; Bremer et al., 1991; Bremer and Aebi, 1992; Orlova and Egelman, 1992; Lorenz et al., 1993; Lepault et al., 1994; Muhlrad et al., 1994; Steinmetz et al., 1997). Also, there is ample evidence that significant structural changes in the actin molecule may occur upon its interaction with actin-binding proteins, including myosin and its fragments (Prochniewicz and Yanagida, 1990; Schwyter et al., 1990; Owen and DeRosier, 1993; McGough et al., 1994; Schmid et al., 1994; Orlova et al., 1995).

**ACTIN IN VIVO**

Feuer et al. (1948) first isolated actin as a major muscle protein. For illustration, in Fig. 2a an ultrathin longitudinal section of a rigor insect flight muscle reveals a MyAclayer (e.g., Taylor et al., 1984) in which thin and thick filaments alternate and are packed with almost crystalline order, thereby maximizing the interaction between the myosin heads radially projecting from the thick filaments and the thin filaments for the development of force (for a detailed review of the more recent history and theories of muscle contraction, see the review of J ontes, 1996). In the 1970s, it slowly but definitely emerged that cytoskeletal actin also plays important roles unrelated to muscle function (reviewed by dos Remedios and Barden, 1993; Way and Weeds, 1990; Pollard, 1986). Accordingly, nonmuscle actin is involved in processes such as cell and intracellular motility, cell division, and dynamic remodeling of the cytoskeleton. Moreover, the bacterial pathogen Listeria monocytogene induces actin polymerization into polar rocket-tail structures within the host cell as a means to promote its rapid intracellular movement and its cell-to-cell transfer (for review, see Southwick and Purich, 1994). As illustrated in Fig. 1e, actin filaments may also serve as molecular "tracks" for myosin motors to move along. Furthermore, F-actin acts as an ATPase, and it interacts with dozens of actin-binding and regulatory proteins and with small effector molecules (see below; for review, see Hennessey et al., 1993, and references therein). Last, but not
Fig. 2. Actin in vivo. (a) Electron micrograph of an ultrathin longitudinal section (for review, see Villiger and Bremer, 1990) through an insect indirect flight muscle (courtesy of Mary C. Reedy, Duke University Medical Center, Durham, NC). The section is one MyAc layer thick and yields an almost crystalline array of alternating thick (i.e., myosin-containing) and thin (i.e., actin-containing) muscle filaments. (b) Goldfish keratocyte labeled with BODIPY-phalloidin. Scales of goldfish were plucked, washed with supplemented medium (amphibian culture medium from Gibco containing penicillin, streptomycin, neomycin, and amphotericin B), and cultured overnight in the same medium in tissue culture flasks. Keratocytes that migrate as a single-cell tissue off the scale were separated from contaminating cells and debris by washing them several times with Ca\(^{2+}\)-free phosphate-buffered saline (PBS). To dissociate the cells and to detach them from the plastic substratum of the tissue culture flask, they were incubated with 5 mM EGTA/5 mM EDTA in Ca\(^{2+}\)-free PBS at pH 7.0 for a few minutes. The exact time required for quantitative release of the cells was determined by observing the tissue culture flask under the microscope. The medium containing the cells was then transferred into centrifuge tubes, the cells were spun down, and the pellet was resuspended in supplemented medium and plated on coverslips, where the cells reattached within minutes. ON, orthogonal network at the front of the cell; EN, endoplasm, the vesicle-rich part of the cytoplasm that contains the nucleus; FC, focal contacts; RF, retraction fibers. (c) Electron micrograph of a human KD fibroblast which has been cultured directly on a Formvar-coated EM gold grid prior to detergent extraction/chemical fixation followed by negative staining with 0.75% uranyl formate. Three distinct cytoskeletal systems can be distinguished: actin-containing microfilaments (MF), vimentin-type intermediate filaments (IF), and tubulin-containing microtubules (MT). Along the cell cortex the microfilaments tend to laterally associate into filament bundles, called stress fibers (SF). Scale bars, 100 nm (a), 10 µm (b), and 250 nm (c).
least, actin filaments may also play a role in compartmentalizing metabolic pathways within cells (for a review, see e.g., Matsudaira, 1991).

The fluorescence micrograph of a goldfish keratocyte shown in Fig. 2b illustrates that the actin cytoskeleton defines the overall size and general shape of this highly motile cell. The actin filaments in the front of the keratocyte's lamellipodium are well organized and form an approximately orthogonal network (ON). Filament length decreases and filament number increases from the endoplasm that contains the nucleus (EN) toward the front end of the lamellipodium. Whereas few long filaments extend all the way through the lamellipodium, most shorter filaments are located at its front end (see also Rinnerthaler et al., 1991; Small et al., 1993). This observation bears interesting consequences for evaluating possible models of the keratocyte's motility that were recently discussed by Small and co-workers (Small et al., 1995). According to these models, actin filaments bind to focal contacts (FC) at the cell's lower surface, thereby mediating adhesion to the substratum, e.g., the culture dish (Small, 1988). Hence, migrating cells exert force on and pull themselves along these focal contacts. Incomplete release of filaments from these contacts frequently occurs, thus resulting in the formation of retraction fibers (RF) at the posterior end of migrating cells.

A diagnostic feature of the actin cytoskeleton of fibroblasts grown in culture is the so-called stress fiber system that traverses the cell near the plasma membrane facing the substratum. Stress fibers are loose bundles of actin-containing microfilaments probably stabilized and/or held together by bundling or crosslinking proteins. Stress fibers are typically 50–500 nm wide and 5–10 µm long. As illustrated in Fig. 2c, in the negatively stained whole mount of a cultured human KD fibroblast, three distinct cytoskeletal filament systems can be distinguished: actin-containing microfilaments (MF), vimentin-type intermediate filaments (IF), and tubulin-containing microtubules (MT). Within the cell unaggregated microfilaments form a loose meshwork, whereas along the cell cortex they bundle into stress fibers (SF) which frequently appear to be associated with microtubules.


Actin has long resisted crystallization since conditions typically favoring protein crystallization cause actin to polymerize into filaments. However, adding stoichiometric amounts of Gd\(^{3+}\) to actin under polymerizing conditions yields 2-D crystals which allowed determination of its 3-D structure to 1.5 nm resolution from tilted views of negatively stained sheet preparations (Aebi et al., 1980, 1981; Smith et al., 1983; see also Fig. 6 and below). Ten years later, the 3-D structure of the actin molecule was resolved to atomic resolution by X-ray diffraction analysis of 3-D crystals of actin in complex with DNase I (Kabsch et al., 1990), gelsolin segment 1 (MacLaughlin et al., 1993), or profilin (Schutt et al., 1993). The atomic structure of the actin molecule determined from the actin–DNase I cocryystals (Kabsch et al., 1990) is displayed in Fig. 3a in the form of a folded ribbon representation. As indicated in Fig. 3b, the entire molecule fits into a box measuring 5.5 × 5.5 × 3.5 nm (cf. Kabsch et al., 1990; Bremer and Aebi, 1992). Each actin subunit is composed of four subdomains, termed 1, 2, 3, and 4. The four subdomains are primarily held together and stabilized by salt bridges and hydrogen bonds between the phosphate groups of the bound nucleotide (i.e., ATP or ADP) and its associated divalent cation, which is believed to be Mg\(^{2+}\) under physiological conditions. The nucleotide and its associated divalent cation are localized to the "heart" of the actin molecule (see Figs. 3a and 3b). The actin subunit is also distinctly polar: subdomain 2 has significantly less and subdomain 1 significantly more mass than the other two subdomains (see Fig. 3b, where the size of the subdomains corresponds to their relative masses).

Using the atomic structure of G-actin, atomic models of the F-actin filament were constructed and refined so as to minimize the difference of their computed diffraction patterns with X-ray fiber diffraction patterns to about 8 Å resolution recorded from oriented gels of F-actin filaments (Holmes et al., 1990; Lorenz et al., 1993; Holmes, 1995). Without question these atomic filament models have had a significant impact on our present understanding of actin filament structure and dynamics. Nevertheless, 3-D helical reconstructions computed from electron micrographs of both negatively stained (e.g., Aebi et al., 1986; Bremer et al., 1991, 1994; see Fig. 8a) and frozen hydrated (Milligan et al., 1990) F-actin filaments have revealed a remarkable similarity to the atomic models when the latter are represented as electron density maps contoured at comparable resolution (i.e., 2.5 nm; e.g., Holmes and Kabsch, 1991; Bremer and Aebl, 1992; Bremer et al., 1994; Hoenger and Aebi, 1996). Moreover, a recent reconstruction of Bremer et al. (1994) allowed interpretation of particular structural features at the level of distinct secondary structural elements, so that predictions based upon the atomic models could be verified (see Fig. 8d and below). Hence, EM-based filament reconstructions have become an invaluable complement to atomic models, particularly in the context of investigations trying to assess distinct
conformational states of the actin polymer in response to various effectors (e.g., Bremer et al., 1991; Orlova and Egelman, 1992, 1993, 1995; Lepault et al., 1994; Steinmetz et al., 1997) or to determine the 3-D structure of actin filaments in complex with actin-binding or regulatory proteins and drugs (e.g., Milligan et al., 1990; Bremer et al., 1991; Owen and DeRosier, 1993; McGough et al., 1994; Schmid et al., 1994; Orlova et al., 1995; McGough and Way, 1995; Steinmetz et al., 1997).

Various aspects of a generalized molecular model of an F-actin filament are presented in Figs. 3d to 3f. For comparison and critical evaluation of the molecular filament model shown in Fig. 3d, Fig. 3c displays a helically filtered image (i.e., D(Z, k)-filtered; cf. Smith and Aebi, 1974; Bremer et al., 1991, 1994) of a negatively stained synthetic F-actin filament stretch such as shown in Fig. 1b. The F-actin filament can be described as consisting of either two right-handed helices of a relatively long pitch (Fig. 3e) or of a single left-handed helix of a shallow pitch (Fig. 3f). Taking into consideration that the intersubunit contacts along the two long-pitch helical strands (right panel of Fig. 3e) are continuous and approximately three times stronger than those between them (Erickson, 1989; for reviews, see Bremer and Aebi, 1992; Holmes and Kabsch, 1991), description of the actin filament as a two-stranded helical polymer such as that shown in Fig. 3e makes most physical sense. Supporting this intersubunit contact pattern, locally separated, unraveled long-pitch helical strands yielding “bubbles” and “splayed ends” have been reported (see Fig. 3h; Aebi et al., 1986; Bremer et al., 1991; Bremer and Aebi, 1992). Both the relative physical strength of the two types of intersubunit contacts and the filament unraveling imply that the two long-pitch helical strands can move relatively independently of each other by “lateral slipping” (Bremer et al., 1991), a mechanism which involves both a radial and an angular component (Censullo and Cheng, 1993; see also below). Nevertheless, the one-start helix description (see Fig. 3f) of the F-actin filament yields a simple parametrization of the filament geometry in terms of a “helical selection rule” which, in turn, allows elegant 3-D helical reconstruction to be performed (see also below; cf. Klug and DeRosier, 1966).

A comparison of the two helix geometries is presented in Figs. 3e and 3f. In the two-start helix model, there are 13 subunits per right-handed long-pitch helical turn of each of the two strands (Fig. 3e, right panel). Assuming an axial subunit repeat of 5.5 nm, 13 subunits yield a pitch of 13 \times 5.5 \text{ nm} = 71.5 \text{ nm}. As a consequence, they cross each other regularly every half pitch, i.e., every 35.75 nm. Figure 3e also reveals the two long-pitch helical strands to be axially staggered by half the axial subunit spacing (i.e., 2.75 nm). The alternative description is as a 5.9-nm pitch one-start “genetic” helix with 13 subunits per 6 left-handed turns (Fig. 3f, right panel). The schematic view in Fig. 3f reveals that assuming a subunit spacing of 2.75 nm, 13 subunits in 6 turns correspond to 13 \times 2.75 \text{ nm} = 35.75 \text{ nm}, i.e., the crossover spacing of two long-pitch helical strands (for reviews, see Holmes and Kabsch, 1991; Bremer and Aebi, 1992).

Asymmetric subunits assemble into intrinsically polar F-actin filaments by always adding onto the filament ends in the same orientation with respect to the filament axis. This filament polarity can be visualized by stoichiometric binding of myosin S-1 to F-actin in situ or in vivo, thereby yielding decorated

**Fig. 3.** The domain structure of the G-actin monomer and the molecular architecture of the F-actin filament. (a) Folding of the actin molecule represented by ribbon tracing of the \(\alpha\)-carbon atoms. An ATP molecule with its associated Ca\(^{2+}\) atom are shown in a Van der Waals sphere representation. (b) Schematic representation of the G-actin molecule in terms of its domain/subdomain organization. At first glance, the actin molecule as shown in (a) appears to be built of two domains, a “small” domain (i.e., containing boxes 1 and 2) and a “large” domain (i.e., containing boxes 3 and 4). At a closer look, both the small and the large domains can be further divided into two subdomains each, called subdomains 1 and 2 (i.e., boxes 1 and 2) and subdomains 3 and 4 (i.e., boxes 3 and 4), respectively. (c) D(Z, k)-filtered image of a negatively stained synthetic F-actin filament stretch such as shown in Fig. 1b. (d) Generalized structure of the F-actin filament built from the actin monomer model displayed in (b). The polarity of the actin filament as revealed by the distinct arrowhead pattern upon stoichiometric decoration with myosin S-1 (S-1) (see and Fig. 5a) is indicated by short lines (see also c and g). (e) The molecular architecture of the actin filament may be described by two intertwined right-handed long-pitch helical strands of actin subunits. The pitch of these long-pitch helical strands is 71.5 nm with 13 subunits per turn (i.e., each having a 5.5-nm axial extent). Seen in projection, the two strands cross each other every 35.75 nm or every half-pitch (see also c). The axial stagger between the two long-pitch helical strands is 2.75 nm, or one half of the axial extent of a subunit. (f) An alternative description of the molecular architecture of the actin filament is that of a shallow left-handed one-start helix. This so-called genetic helix has a pitch of 5.9 nm, and it contains 13 subunits in 6 left-handed turns, so its helical repeat amounts to 35.75 nm or one crossover of the two long-pitch helical strands (see e). (g) (right) Negatively stained S-1-decorated F-actin filament stretch with its “barbed” end to the left and its “pointed” end to the right (see also Fig. 5a). (left) D(Z, k)-filtered filament projection image such as shown in (c), demonstrating that the “mini-arrowhead” appearance (indicated by short lines) of the undecorated F-actin filament is opposite to the polarity revealed by the S-1 decoration pattern. (h) Gallery of ADP-F-actin filament stretches after negative staining with 1% sodium phosphotungstate, pH 7.0. This heavy metal salt, which acts as an inorganic phosphate analogue (Pi), causes local unraveling of the two long-pitch helical strands so as to produce “bubbles” and “splayed ends” (see arrowheads). Scale bars, 1 nm (a), 50 nm (g), and 100 nm (h).
filaments with an “arrowhead-like” appearance (Moore et al., 1970). The right half of Fig. 3g displays a short stretch of such a negatively stained S-1-decorated F-actin filament with its “barbed” end to the left and its “pointed” end to the right (see also Fig. 5a). The polarity revealed by this S-1 decoration pattern is opposite to that of the “mini-arrowhead” appearance observed in computed EM projection images of negatively stained F-actin filament stretches (Bremer et al., 1991; Bremer and Aebi, 1992), such as that illustrated in the left half of Fig. 3g (compare also with Figs. 3c and 3d). This mini-arrowhead pattern, in turn, can be used to determine the polarity of individual F-actin filaments directly (i.e., without S-1 decoration). For instance, Bremer et al. (1994) have employed this method for aligning individual F-actin filament 3-D reconstructions prior to computing their averaged 3-D reconstructions. Also, Meyer and Aebi (1990), with α-actinin, and Faix et al. (1996), with cortexillin I, have used this direct method to determine whether these F-actin bundling proteins crosslink actin filaments in a parallel or an antiparallel fashion.

TESTING PREDICTIONS OF ATOMIC MODELS

A number of mutant actins, obtained by either selection after random mutagenesis, genetic identification and isolation, or by mutagenesis in vitro have been produced (for review, see Hennessey et al., 1993; Sheterline et al., 1995). They allow probing predictions of atomic models for the structure of the actin filament (e.g., Lorenz et al., 1993) and dissecting the anatomy of the actin molecule functionally both in vivo and in vitro.

Many mutant actins display “antimorphic” or “dominant negative” phenotypes in vivo, i.e., the heterozygous organism exhibits the mutant phenotype. A likely explanation for this observation is that filament formation involves several binding sites on the actin molecule (for review, see Sparrow et al., 1992; Sparrow, 1995), and so, if a point mutation happens to destroy one of them, the remaining binding sites on this molecule may still be functional. Therefore, the mutant actin molecule may nonproductively interact with wild-type molecules in that it still incorporates into filaments made of wild-type molecules but then blocks the addition of further subunits. Because of this antimorphic behavior, in vivo effects of mutant actins have often been difficult to interpret. Fortunately, the Drosophila genome contains one actin gene which can be mutated without producing a lethal phenotype: Act88F encodes actin III, a unique actin isoform expressed solely in the indirect flight muscle (Fyrberg and Donady, 1979; Fyrberg et al., 1983). For in vitro studies, transgenic flies harboring a mutant actin gene can be generated by P-element-mediated germline transformation into an Act88F null mutant (see, e.g., Drummond et al., 1990).

A mutant actin which has been extensively characterized involves a point mutation of Gly245 to Asp245, called G245D. Chemical mutagenesis of the diploid human KD fibroblast strain led to the isolation of the substrain HU14 which is both immortalized and tumorigenic (Leavitt and Kakunaga, 1980; see also Leavitt et al., 1982). The HU14 substrain expresses one G245D and one wild-type β-actin allele each (Vandekerckhove and Weber, 1980). The G245D actin incorporates into stress fibers at lower levels than wild-type actin, and it alters cell morphology (Leavitt and Kakunaga, 1980; Leavitt et al., 1987). Figure 4a documents that G245D actin is also polymerization deficient in vitro: The lane marked “Start” reveals the actin mixture isolated from tumors induced in nude mice after inoculation with HUT-14 cells. The lane marked “Pel” contains the pellet of the polymerized actin mixture, whereas the lane marked “Sup” contains the corresponding supernatant. The G245D actin clearly enriches over the wild-type actin in the supernatant (i.e., it is not efficiently polymerized), whereas the wild-type actin is almost quantitatively recovered in the pellet. Hence, G245D actin can selectively be enriched from a mixture of wild-type and mutant actin by “cycling” (i.e., repeated polymerization and depolymerization of high-speed supernatants). Figure 4b demonstrates that upon addition of e.g., 2 mM MgCl₂/50 mM KCl to G245D mutant actin, instead of polymerizing into filaments (see inset), it associates into unspecific, loose aggregates. This clearly demonstrates that actins mutated at Gly245 (i.e., G245D or G245K) exhibit a serious polymerization defect (Millonig et al., 1988; Taniguchi et al., 1988; Aspenstrom et al., 1992). Consistent with this finding, heterozygous flies being transgenic for G245D actin produce myofibrils that appear normal on eclosion but eventually become disrupted over the following 12–14 days, so that the flies eventually become flightless (Sakai et al., 1991; Sparrow et al., 1992).

Figure 4c shows residues 243–245 (Pro-Asp-Gly) at the tip of subdomain 4 of one subunit and residues 322–325 (Pro-Ser-Thr-Met) at the bottom of subdomain 3 of the subunit above it, specifying one of the major intersubunit contacts along the two long-pitch helical strands. The polymerization-deficient phenotype of G245D actin is therefore not at all surprising: as is documented in Fig. 4c, replacing the Gly residue at position 245 by a negatively charged residue such as Asp is likely to block polymerization by a charge mismatch. As is illustrated schematically in Fig. 4d, G245D mutant actin can, however, still be induced to form “folded ribbons” and sheets
(Millonig et al., 1988). In contrast to F-actin filaments (which are “upper dimer” related), these supramolecular assemblies are “lower dimer” related (see below and Fig. 6c) and involve intersubunit contacts distinct from those engaged in filament formation.

A prominent feature in the atomic model of the F-actin filament (Holmes et al., 1990; refined by Lorenz et al., 1993) is an extended β-hairpin (i.e., residues 262–274). This structure is commonly referred to as the “hydrophobic plug” because the four central residues (266–269) forming its turn are hydrophobic. Holmes et al. (1990) suggested a plug-like insertion of this hydrophobic loop across the filament axis into a complementary “hydrophobic pocket” provided by the interface between two adjacent subunits of the opposite long-pitch helical strand. To test this hypothesis, Rubenstein and co-workers (Chen et al., 1993) mutated a Leu residue, L266, of the yeast ACT1 gene to Asp (L266D), thereby introducing a hydrophilic residue into the hydrophobic plug. L266D actin exhibited a cold-sensitive phenotype with a higher critical concentration for polymerization than wild-type actin. This finding indicated that L266D actin cannot form nuclei at low temperature since the mutated hydrophobic plug can no longer adopt a proper filament conformation. Independent confirmation for Holmes’ hydrophobic loop hypothesis came from 3-D reconstructions of negatively stained F-actin filaments by Bremer et al. (1994) that revealed a distinct high mass density “bridge” across the filament axis that coincided with the position of the hydrophobic plug (Fig. 8d; see also below).

OLIGOMERIZATION, POLYMERIZATION, AND CRYSTALLIZATION OF ACTIN

As documented in Fig. 5c, when monitored by the increase of the fluorescence signal of actin pyrenated at Cys374, the time course for polymerizing G-actin monomers into F-actin filaments is distinctly sigmoidal. It can be divided into at least three distinct phases: an initial nucleation or lag phase, followed by an almost linear elongation phase, and finally, a steady-state phase. Therefore, polymerization is commonly considered a nucleation–elongation process, involving fast monomer activation, rate-limiting nucleation, and moderately fast elongation (reviewed by Pollard, 1990; Carlier, 1991; Estes et al., 1992). The nucleus is defined as the first oligomer formed during polymerization that is more likely to grow than to shrink. For actin polymerization, most available evidence suggests the nucleus to be a trimer (i.e., involving three subunits along the genetic helix; see Figs. 3f and 5e). During the lag phase, monomer activation and nucleus formation predominate, and this process strongly depends on, e.g., the type of divalent cation (Ca$^{2+}$ or Mg$^{2+}$) present at the high-affinity divalent cation-binding site (HAS) of the G-actin molecule (Fig. 11; see also below). After a significant number of nuclei have been formed, rapid subunit addition ensues, and the polymerization reaction progresses into the elongation phase during which the relationship of total polymer versus time is approximately linear. The kinetic rate constants for filament elongation strongly depend on the exact polymerization conditions (e.g., pH, temperature, ionic strength, state of hydrolysis of the bound nucleotide, and type of divalent cation bound to the HAS of G-actin), but an overall elongation rate of roughly 50 subunits per second and filament (i.e., corresponding to about 140 nm/sec filament growth) is probably within the right order of magnitude (see, e.g., Pollard, 1983). Finally, at steady state the monomer and polymer concentrations remain invariant, and the monomer concentration equals the critical concentration for polymerization. However, subunit exchange between the polymer and the monomer pools continues by a phenomenon called “treadmilling” (i.e., subunit flux through the filament). Possibly, at steady state breaking and reannealing of filaments may also occur at significant rates (reviewed by Oosawa, 1993).

As was discussed earlier and is depicted in Figs. 3a and 3b, the 4-domain actin molecule is stabilized by a tightly bound adenine nucleotide (i.e., ATP or ADP) and its associated divalent cation (i.e., Ca$^{2+}$ or Mg$^{2+}$). At first glance, treadmilling seems to contradict the laws of thermodynamics, but the polymerization of actin with bound ATP (i.e., ATP–G-actin) dissipates chemical energy via ATP hydrolysis and is therefore not strictly reversible (Carlier, 1992). Consistent with this finding, it was first shown by Wegner (1976) that an energetic difference existed between the two filament ends. Hence, it is remarkable that ADP–G-actin also polymerizes into filaments, only at an approximately 25-fold higher critical concentration and at a much slower rate than ATP–G-actin (Pollard, 1984). ATP hydrolysis increases the rate of subunit exchange: compared to ATP–G-actin ADP–G-actin dissociates about 10 times faster from the barbed end of the filament (Korn et al., 1987; Carlier and Pantaloni, 1988).

Standard isolation protocols (reviewed in Pardee and Spudich, 1982) yield Ca$^{2+}$–ATP–G-actin (i.e., with Ca$^{2+}$ bound to the HAS of the molecule), whereas in vivo the majority of G-actin is believed to be Mg$^{2+}$–ATP–G-actin (reviewed in Estes et al., 1992). The affinities of actin for both ATP and the tightly bound divalent cation are in the nanomolar range (Estes et al., 1992). Under most polymerization conditions, ATP hydrolysis lags behind polymerization (e.g., Pollard and Weeds, 1984) and because of this uncoupling,
Characterization of a mutant human β-actin, called G245D, involving a point mutation of Gly245 to Asp245. (a) SDS–PAGE of a mixture of wt and G245D actin before and after polymerization with 2 mM MgCl₂ and 50 mM KCl: lane 1 (Start), actin mixture before polymerization; lane 2 (Pel), high-speed (i.e., for 1 hr at 100,000 g) pellet of actin mixture after polymerization; and lane 3 (Sup), high-speed supernatant of actin mixture after polymerization. The enrichment of G245D actin over wt actin in the supernatant is evident, indicating an altered polymerization behavior of G245D actin compared with wt actin. (b) Electron micrograph of G245D actin after polymerization with 2 mM MgCl₂ and 50 mM KCl, negatively stained with 0.75% uranyl formate. For comparison, wt actin polymerized and prepared for EM in the same way is shown in the inset. (c) Interface (left, for wt actin; right, for G245D actin) of the actin–actin contact along the long-pitch helical strands (see also Figs. 3e, 8b, and 8c) at the tip of subdomain 4 (i.e., involving residues P243, D244, and G/D245) of one subunit and subdomain 3 (i.e., involving residues P322, S323, T324, and M325) of the next subunit above it. The atomically built models for wt and G245D actin illustrate that replacing the Gly by the larger Asp side chain at amino acid residue 245 is likely to cause a charge mismatch within the corresponding actin–actin contact along the long-pitch helical strands, thereby interfering with polymerization into F-actin filaments. (d) Schematic drawing illustrating that while G245D actin fails to polymerize into bona fide F-actin filaments (see b), it remains capable of forming 2-D crystalline arrays in the presence of stoichiometric amounts of Gd³⁺ (see Figs. 6 and 7; also, Millonig et al., 1988). Scale bar, 100 nm (b).
the state of hydrolysis of the bound nucleotide within the newly formed actin filaments changes with time. Since ATP hydrolysis is fast and irreversible and the release of inorganic phosphate (P$_i$) is slow and reversible, neither ATP– nor ADP–F-actin but ADP–P–F–actin is the major intermediate present during polymerization.

Since actin has been the prototype of a protein readily polymerizing into polar filaments (Fig. 5a), it came somewhat as a surprise that at least in vitro it could also form a variety of nonfilamentous polymer or crystalline supramolecular assemblies (Aebib al., 1981), for example, crystalline actin tubes, as shown in Fig. 6a. Along the same lines, specific and stoichiometric interaction with actin-binding proteins does interfere with actin’s structural polymorphism: depending on the exact ionic conditions, stoichiometric mixtures of actin and DNase I will form either actin–DNase I co-crystals (Mannherz et al., 1977; reviewed in Mannherz et al., 1992) or actin–DNase I tubes (Fig. 7e; Fowler et al., 1984).

Moreover, it has become evident that a significant fraction of G-actin dimerizes at early stages of polymerization under a wide variety of polymerization conditions (see Newman et al., 1985; Goddette et al., 1986; Matsudaibra et al., 1987; Millonig et al., 1988; for review, see Grazi, 1989). Compared to the very careful analyses of monomer activation, nucleation, elongation, and the effects that the nucleotide and the divalent cation bond to the HAS have on polymerization rate constants, this dimerization step went largely unnoticed. Figure 5b depicts a time course of actin polymerization monitored by covalent crosslinking with N,N’-1,4-phenylenebismaleimide (1,4-PBM) (Knight and Offer, 1978). As polymerization proceeds, an initially formed 86-kDa apparent molecular weight dimer (i.e., the so-called “lower dimer,” LD) is gradually consumed concomitant with the formation of a 130-kDa apparent molecular weight dimer (i.e., the so-called “upper dimer,” UD) (Millonig et al., 1988; Steinmetz et al., 1997). Biochemical quantities of covalently crosslinked LD and UD have been purified, and analytical ultracentrifugation has verified that both species are in fact dimers (Millonig et al., 1988). According to protein chemical analysis, Lys191 of one subunit is crosslinked to Cys374 of another subunit in the UD (Elzinga and Phelan, 1984). The LD crosslink most likely occurs between Cys374 of both subunits (Millonig et al., 1988). The two covalently crosslinked actin dimer species are drawn schematically in Fig. 5d, illustrating that the LD can migrate very similarly to a polypeptide chain of twice the molecular mass of an actin monomer by SDS–PAGE, whereas the UD cannot adopt a relaxed linear conformation and hence exhibits an anomalous mobility by SDS–PAGE.

LD and UD were assayed for their ability to nucleate actin polymerization, to polymerize into filaments, and to form paracrystalline and crystalline actin arrays (Millonig et al., 1988). As illustrated schematically in Fig. 5e, these experiments have suggested the following assembly pathways: As a first and fast step, actin monomers form LD at least as an intermediate under polymerizing conditions. LDs might assemble further into crystalline arrays such as sheets (see below and Fig. 7) or they might dissociate again into monomers once the amount of free monomer has dropped below its critical concentration for polymerization. In parallel and more slowly, actin monomers form UD and larger oligomers (trimers, . . . ) which efficiently nucleate F-actin filament polymerization. Interconversion between LD-based and UD-based supramolecular assemblies has been observed (e.g., Aebi et al., 1981; Smith et al., 1983), but it is unclear how this interconversion occurs. As indicated schematically in Fig. 6c, while being incompatible with the molecular architecture of the F-actin filament the LD may be shared among adjacent filaments (i.e., act as an interfilament dimer) in actin filament bundles or paracrystalline filament arrays (Millonig et al., 1988).

Using EM, we have recently documented that during the process of filament elongation LD might...
directly be incorporated into growing F-actin filaments via one of its subunits. Gradually, the surplus monomers dissociate from these “LD-decorated” filaments (probably upon switching of the incorporated LDs from a G-like to an F-like conformation), thereby yielding bona fide mature F-actin filaments at steady state (Steinmetz et al., 1997). These data strongly suggest that actin polymerization may involve multiple pathways rather than just the commonly assumed nucleation–elongation mechanism (see above). Based on these recent findings, LD appears to be an important intermediate in the actin polymerization pathway and should therefore be considered in any future models trying to describe this process. Interestingly, recent in vitro studies with gelsolin, actin-binding, and swinholide A revealed that these three actin-binding molecules were able to stabilize an LD-type actin dimer (Hesterkamp et al., 1993; Bubb et al., 1994a,b, 1995), thus further supporting the functional significance of the LD in F-actin filament polymerization and turnover (Steinmetz et al., 1997; see also above). In this context, one may speculate that during dynamic reorganization or turnover of the actin cytoskeleton, a substantial amount of actin is present in the form of an LD pool in the cell. Moreover, incorporation of LD into growing F-actin filaments might be important in building an actin meshwork that can rapidly assemble and disassemble in highly motile cells and which might be a prerequisite for the concerted action of actin-crosslinking proteins.

Double-layered actin sheets (Smith et al., 1983) and actin tubes (Fig. 6; Steinmetz et al., in preparation) are closely related 2-D crystalline actin arrays (Fig. 7f) which form in the presence of stoichiometric amounts of the trivalent lanthanide gadolinium. In both cases, the unit cell of the respective 2-D crystals contains two actin monomers which are related by a P2 symmetry axis normal to the crystal plane. Currently, we are determining the actin–actin interactions within the crystalline tubes at atomic scale by merging the atomic structure of the G-actin monomer (Kabsch et al., 1990) with an EM-based 3-D reconstruction of the actin tubes (Fig. 6b). In this context, we are also investigating the possibility of a structural relationship of the actin–actin contacts within the sheet or tube dimer with the conformation of the LD which forms predominantly at the onset of actin polymerization (see above and Fig. 5c; also Millonig et al., 1988) and may incorporate with one of its subunits into growing F-actin filaments (Steinmetz et al., 1997).

Actin-binding proteins have been designed to modulate actin polymerization, actin filament length, and the steady-state concentrations of monomer and polymer in vivo (reviewed in Pollard and Cooper, 1986; Condeelis, 1993; Kreis and Vale, 1993; Stossel, 1993; Hitt and Luna, 1994; Rozycki et al., 1994). Most nonmuscle cells typically contain on the order of approximately 20 mg/ml actin. Driven by the ionic strength of the cytoplasm (i.e., 1–2 mM Mg$^{2+}$ and 75–150 mM K$^+$), this amount of actin should be quantitatively polymerized into F-actin filaments. Instead, typically 20–50% of the cytoplasmic actin is present in an unpolymerized form in nonmuscle cells. Monomer-binding proteins (e.g., DNase I) lower the effective G-actin concentration by sequestering monomers, thereby causing depolymerization of actin filaments. Some actin-binding proteins also catalyze exchange of ADP for ATP (e.g., profilin) or have a higher affinity for ATP–G-actin (e.g., thymosin β4). Usually, proteins that bind stoichiometrically to actin filaments stabilize them (e.g., tropomyosin, nonmuscle desmin).

Most capping proteins (e.g., capZ) bind to the barbed end of F-actin filaments (see Fig. 5a), thereby slowing down polymer turnover. This, in turn, limits the average filament length and hence the local viscosity of the corresponding filament meshwork. Moreover, it increases the critical concentration for polymerization (which is higher at the pointed end). Severing proteins (e.g., gelsolin) fragment actin filaments and bind to the barbed end of one of the fragments, thereby having the same multiple effects as do capping proteins (see above). In addition, they accelerate depolymerization by increasing the number of
pointed ends. Most severing proteins can sever phalloidin-stabilized actin filaments; however, actophorin does not (Maciver et al., 1991). F-actin capping and severing proteins can also stabilize nuclei and thus increase the number of filaments that are formed.

3-D STRUCTURE, DYNAMICS, AND MECHANICAL PROPERTIES OF F-ACTIN FILAMENTS: FACTS AND FICTIONS

The structural disorder of F-actin is manifested by the variable crossover spacing and filament diameter along its length and by the local unraveling of the two long-pitch helical strands (see Fig. 3h and above). This structural disorder has been the subject of many investigations and is now generally accepted to represent an intrinsic dynamic property of the F-actin filament. To formally describe this dynamic behavior of the actin polymer, two models have been proposed: (1) “random twist with cumulative angular disorder” (Egelman et al., 1982; Stokes and DeRosier, 1987) and (2) “compensatory lateral slipping” (Bremer et al., 1991), with the lateral slipping motion of the two long-pitch helical strands having both a radial and an angular component (Censullo and Cheung, 1993). We have found that the length and corresponding maximum width of single crossovers determined from STEM ADF images of negatively stained F-actin filaments (Fig. 1b) appear uncorrelated for many polymerization conditions assayed including stabilization by phalloidin (Steinmetz et al., 1997). These findings are in accord with the model of Bremer et al. (1991), and they further indicate that cumulative lateral slipping may indeed occur locally over short filament stretches (i.e., 2–6 subunits). However, over longer filament stretches local disorder or distortions compensate each other so that there is no net propagation of structural or mechanical perturbation along an actin filament. Hence, we have to conclude that our findings do not support the results of Egelman and co-workers which proposed allostery and long-range cooperativity to occur in F-actin filaments (Egelman and Orlova, 1995a,b), for example, caused by single gelsolin molecules upon binding to the barbed end of a filament (Orlova et al., 1995; Prochniewicz et al., 1996).

To further investigate the structural basis of F-actin dynamics, we have critically assessed the fidelity and significance of a refined and averaged 3-D reconstruction (i.e., a “consensus” reconstruction) produced from a set of F-actin filaments polymerized from Ca-ATP-G-actin with 2 mM MgCl₂ and 50 mM KCl. Specifically, we have investigated in detail how resolution and suboptimal data processing steps may affect distinct structural features of 3-D reconstructions computed from negatively stained F-actin filaments (Bremer et al., 1994). The refined and averaged consensus reconstruction from Bremer et al. (1994) is displayed in Fig. 8a surface-rendered so as to include 100% (left) or 30% (right) of its nominal molecular mass. In Figs. 8b to 8d, we have aligned this consensus reconstruction with a ribbon representation of the atomic model by Holmes et al. (1990): the fit is remarkable. Among other structural features, this reconstruction strongly supports the prediction of Holmes et al. (1990) and Lorenz et al. (1993), who modeled the hydrophobic plug (see arrows in Fig. 8c) to extend across the filament axis so as to insert in a plug-like fashion into a hydrophobic pocket provided by the interface between two adjacent subunits of the opposite long-
Actin polymorphism (for details see Millonig et al., 1988). CTEM images of negatively stained samples of various polymeric or crystalline in vitro assemblies induced from rabbit muscle actin. (a) Bonafide F-actin filaments; (b) actin filaments made of LD instead of UD; (c) tube-like assemblies induced from an ~35-kDa proteolytic fragment of actin; (d) folded ribbons made of actin LD; (e) stacked-disk-type actin-DNase I tubes (cf. Fowler et al., 1984); and (f) a mixture of crystalline actin sheets and tubes (cf. Aebi et al., 1981; see also Fig. 6a). Scale bar, 100 nm (a–f). Reproduced from J. Cell Biol. 106, 785–796 by copyright permission of The Rockefeller University Press.
pitches helical strand. When contoured to include 30% mass, our consensus filament reconstruction reveals a distinct mass density every 2.75 nm which connects the two long-pitch helical strands laterally like the "rungs" of a ladder (Figs. 8a and 8d). Remarkably, the hydrophobic loop coincides with this distinct "bridge" of high mass density (Fig. 8d) which emanates from the region close to the interface between subdomains 3 and 4 (Fig. 8d, arrows). To explain our observation of local unraveling (see Fig. 3h) within a double-stranded filament where hydrophobic loops emanate at regular intervals from one long-pitch helical strand and insert into hydrophobic pockets provided by the other strand, a molecular "zipper" model is best suited.

It is conceivable that different polymerization conditions may modulate the inherent structural dynamics of the F-actin filament or shift equilibria between distinct structural/functional states and thus change the overall filament conformation. Such conformational changes are in principle observable by EM. However, different polymerization conditions may produce "better" or "worse" looking filaments in terms of how suitable they are for 3-D helical reconstruction. Therefore, investigation of subtle conformational changes requires a careful analysis of the quality of the data and of the reliability of the processing steps applied, to exclude artifacts which may simply be caused by different effective resolution of the individual data sets. We addressed this issue by taking advantage of the increased contrast, the better signal-to-noise ratio, and the linear contrast transfer characteristics of STEM ADF over CTEM BF images (Engel and Colliex, 1993) of negatively stained specimens (Fig. 1b) (Bremer et al., 1994). Obviously, the phenotypes of the individual refined reconstructions which go into an averaged reconstruction do affect the phenotype of the average: Figs. 9a and 9b display the 10 three-crossover long filament stretches that were included in the refined and averaged consensus reconstruction by Bremer et al. (1994): they have been surface-rendered to include 100% (a) or 30% (b) of their nominal molecular mass. In Fig. 9b, these 10 refined reconstructions have been classified according to the strength of their intersubunit contacts between the two long-pitch helical strands: the type A reconstructions reveal little if any connectivity, the type B reconstructions yield interrupted connectivity, and the type C reconstructions exhibit uninterrupted connectivity between the two long-pitch helical strands. It is worth mentioning that most of the individual reconstructions look quite similar when contoured to include 100% mass: in Fig. 9a, compare, e.g., the type A filament reconstructions with those of the type C. As pointed out above, producing averages of the different filament types (Fig. 9c: ΣA, ΣB, and ΣC) yields subaverages that accentuate the structural features which were initially chosen on the basis of the overall average (Fig. 9c: Σ10) as "earmarks" for classification. As with the individual reconstructions (compare Fig. 9a with Fig. 9b), the significant differences observed for the averages of the different filament types at the 30% contouring level (Fig. 9c), however, become almost invisible at the 100% contouring level (Fig. 9d).

In addition, we have systematically investigated the phenotypes of reconstructions that are produced upon deviation from the optimal helical parameters or by lowering the nominal resolution of the reconstruction (cf. Bremer et al., 1994). For example, as revealed in Fig. 10a, small deviations from the optimal radial position of the helix axis (i.e., 0.1–1.0 nm) yield a substantial redistribution of mass from several parts of the filament surface into the area between the two long-pitch helical strands (i.e., see buildup of a high mass density "bridge" connecting the two long-pitch helical strands marked by an arrow). Hence the detailed morphology of the intersubunit contact pattern between the two long-pitch helical strands is extremely sensitive to small deviations from the optimal radial coordinate of the filament axis. In fact, it is remarkable that a shift as little as 0.06 nm of the radial coordinate of the

**Fig. 9.** Variations of the intersubunit contact pattern among different F-actin filaments. (a) 3-D reconstructions of 10 three-crossover long F-actin filament stretches surface-rendered to include 100% of their nominal molecular mass are displayed and enumerated 1 through 10. (b) The same 10 reconstructions are shown surface-rendered to include 30% of their nominal molecular mass, and they have been classified into three distinctive types of reconstructions according to their intersubunit contact pattern: Type A exhibits strong long-pitch helical intersubunit contact and little if any contact between the two strands. Type B reveals continuous strong contact along and interrupted contact between the two long-pitch helical strands. Finally, Type C yields predominant contact between the two strands. (c) The reconstructions within each of the three types A, B, and C shown in (b) were averaged, and the resulting subaverages ΣA, ΣB, and ΣC are compared to the overall average Σ10, all surface-rendered to include 30% of the nominal molecular mass. Arrows identify areas that clearly differ among the subaverages besides the relative mass density of the intersubunit contacts between the two long-pitch helical strands (marked by arrowheads) that was used to classify the reconstructions in the first place. (d) The subaverages ΣA, ΣB, and ΣC and the overall average Σ10 are displayed surface-rendered to include 100% of the nominal molecular mass. In Σ10, the arrowhead indicates the interdomain cleft that separates the larger inner (i.e., subdomains 3 and 4) from the smaller outer (i.e., subdomains 1 and 2) domain (see Figs. 3a and 3b), whereas the arrow points at the interstrand contact interface. This figure has been adapted from Figs. 4a and 5 of Bremer et al. (1994).
FIG. 10. Phenotypes of F-actin filament reconstructions resulting from deviations from the optimal helical parameters or from different nominal resolutions (for details, see Bremer et al., 1994). (a) Effects of deviating from the optimal radial coordinate of the filament axis on the phenotype of the corresponding F-actin filament 3-D reconstructions. For this purpose, the 10 three-crossover long F-actin filament stretches (see Fig. 9a) have been reconstructed with the filament axis radially shifted as indicated, aligned and averaged, and are compared to the optimal reconstruction $S_{10}$ as displayed in Figs. 8a and 9d. The arrow highlights the connectivity between subdomain 4 of one subunit from one long-pitch helical strand with subdomain 1 of the closest subunit from the other strand. The arrowhead points to a region of the interstrand interface where structural detail deteriorates when radially displacing the filament axis from its optimal position. (b) Effect of lowering the nominal isotropic resolution on the phenotype of the F-actin filament 3-D reconstructions. The nominal resolution of $\Sigma_{10}$ as shown in Figs. 8a and 9d has been reduced from 2.5 to 3.5 or 4.5 nm as indicated. Whereas the arrows highlight the same feature as in (a), the arrowheads point to the interdomain cleft. All reconstructions in (a) and (b) are shown surface-rendered to include 100% (top in a, bottom in b) or 30% (bottom in a, top in b) of the nominal molecular mass. This figure has been adapted from Fig. 6 of Bremer et al. (1994).
filament axis (i.e., within a 3-D reconstruction to a nominal resolution of 2.5 nm) yields distinct changes of the corresponding reconstruction (see Fig. 10a). Moreover, lowering the nominal resolution has a similar effect but, at the same time, it weakens the long-pitch helix intersubunit contact (see arrow in Fig. 10b). In addition, lowering the resolution alleviates the cleft between the large and the small domains of the actin subunit (see arrowhead in Fig. 10b).

We have also assessed the effect of different divalent cations (i.e., Mg$^{2+}$ or Ca$^{2+}$) bound to the high-affinity divalent cation binding site (HAS) of the G-actin molecule on its polymerization kinetics and the oligomers that form upon addition of salt, as well as the mechanical properties and 3-D structure of the resulting F-actin filaments (Steinmetz et al., 1997). As illustrated in the left panel (top) of Figs. 11a and 11b, monitored by the increase of the fluorescence signal of pyrenated actin (i.e., at Cys374), the lag phase of Ca–ATP–G-actin appears much more pronounced than that of Mg–ATP–G-actin, while the elongation rate remains similar for both conditions. Both low-magnification views (i.e., middle panels (top left) of Figs. 11a and 11b) as well as high-magnification views (i.e., middle panels (top right) of Figs. 11a and 11b) document that the gross morphologies of the two types of F-actin filaments are very similar. The flexibility of the actin filaments was determined from the average crossover spacing according to Bremer et al. (1991) (i.e., middle panels (bottom left) of Figs. 11a and 11b), and the apparent persistence length was measured according to Orlova and Egelman (1993) (i.e., based on Landau and Lifshitz (1958); see middle panels (bottom right) of Figs. 11a and 11b). These data clearly document that the type of divalent cation bound to the HAS of G-actin does not significantly affect the mechanical properties of the resulting F-actin filaments, a finding being consistent with results derived from thermal fluctuations of F-actin filaments in solution (Isambert et al., 1995). These results are strongly supported by our refined and averaged 3-D reconstructions of corresponding F-actin filaments. As illustrated in the right panels of Figs. 11a and 11b, no significant structural differences were revealed between Mg– and Ca–F-actin (for details, see Steinmetz et al., 1997). In contrast, Egelman and co-workers (Orlova and Egelman, 1993; Egelman and Orlova, 1995b) aimed to document that F-actin flexibility can be increased up to four times, depending on the ionic conditions applied. Also, their corresponding 3-D helical reconstructions revealed substantial structural differences, a finding which our data failed to confirm (right panels of Figs. 11a and 11b).

We have also investigated the effect of phalloidin on actin polymerization. For this purpose, Ca–ATP–G-actin was polymerized with 100 mM KCl in the presence of a 2:1 molar excess of phalloidin over actin (Fig. 11c). As documented by the time course of the pyrene fluorescence increase in the left panel (top) of Fig. 11c, phalloidin drastically shortens the lag phase of Ca–ATP–G-actin, and it accelerates the elongation rate by a factor of about 2 (compare with Fig. 11b). Filaments polymerized from Ca–ATP–G-actin in the presence of phalloidin (Fig. 11c, middle and right panels) differ from their native counterparts (compare with Fig. 11b) mainly in three aspects (for details and discussion, see Steinmetz et al., 1997): (1) As revealed by the middle panel of Fig. 11c, their mean crossover spacing increases by 2 nm (bottom left), and their apparent persistence length more than doubles (bottom right). (2) As marked by the arrowhead in the right panel of Fig. 11c (filament reconstruction contoured to include 100% mass), the distinct “groove” residing between the two long-pitch helical strands becomes “padded” with additional mass. And (3) as indicated by the two arrowheads in the right panel of Fig. 11c (filament reconstruction contoured to include 30% mass), the intersubunit contacts both along and between the two long-pitch helical strands become more massive. Our EM-based 3-D reconstructions of phalloidin-stabilized Ca–F-actin filaments (see also Steinmetz et al., 1997) are in good qualitative agreement with a corresponding atomic model computed by Lorenz et al. (1993). Lorenz et al. (1993) also determined the possible binding site of phalloidin within their atomic filament model. Their placement of the toxin molecule is in agreement with its EM-based localization (Bremer et al., 1991; refined by Steinmetz et al., 1997; see also below), its chemical mapping (Vandekerckhove et al., 1985), and its localization predicted by mutational analysis (Drubin et al., 1993).

Recently, we have engineered an undecagold-tagged phalloidin derivative (Au$_{11}$-phalloidin; in collaboration with Dr. H. Faulstich, Max-Planck Institute for Medical Research, Heidelberg, Germany) in order to directly map the binding site of this bicyclic heptapeptide within the F-actin filament (Steinmetz et al., manuscript in preparation). As documented in Fig. 12a, the ~1-nm-diameter gold clusters can be visualized directly by STEM ADF of unstained freeze-dried Au$_{11}$-phalloidin-stabilized F-actin filaments. Remarkably, the gold particles are lining up the two long-pitch helical strands at distinct, 5.5-nm intervals and are axially spaced by 2.75 nm (i.e., due to the 2.75-nm axial stagger of the two long-pitch helical strands). At first glance, a refined and averaged 3-D reconstruction computed from STEM ADF images of negatively stained Au$_{11}$-phalloidin-stabi-
lized F-actin filament stretches (Fig. 12b) looked indistinguishable from a corresponding 3-D reconstruction computed from just phalloidin-stabilized F-actin filaments (see Fig. 11c, right panel). However, a difference map computed from these two filament reconstructions after proper scaling yielded a single positive mass peak per actin subunit on the filament surface. In Fig. 12b, the difference peaks are shown mapped onto the refined and averaged Au$_{11}$-phalloidin-stabilized F-actin filament 3-D reconstruction as 1-nm-diameter gold spheres. As the Au$_{11}$-cluster is attached to the bicyclic heptapeptide via a ~1.7-nm-long linker, the gold particles are expected to appear displaced from the actual phalloidin-binding sites along the actin filament. Taking the location of the phalloidin molecule within the F-actin filament as proposed by Lorenz et al. (1993), we have fitted an atomic model of the undecagold-phalloidin complex into our EM-based Au$_{11}$-phalloidin-stabilized F-actin filament 3-D reconstruction. Whereas Lorenz's orientation of the phalloidin molecule caused the attached gold cluster to sterically collide with an adjacent actin molecule, rotation of the phalloidin moiety by approximately 180° about an axis roughly parallel to the filament axis yielded and almost perfect coincidence of the gold cluster with the unique difference peak such as shown in Fig. 12b.

In summary, we have optimized our F-actin filament preparation and EM data acquisition, and we have improved the fidelity and reproducibility of our semiautomated helical data processing protocol. Taken together, these steps have become a powerful and valuable tool, particularly to more systematically assess distinct conformational states of the F-actin polymer in response to various effectors (e.g., the type of the tightly bound divalent cation, the state of hydrolysis of the bound nucleotide, and the binding of small drugs such as phalloidin, etc.) or to more faithfully determine the 3-D structure of actin filaments in complex with actin-binding or regulatory proteins and molecules. 

CONCLUDING REMARKS

Our recent advances in specimen preparation, imaging techniques, and data analysis, processing, and merging now open exciting new avenues toward a more profound understanding of the structural basis and the complex mechanisms which underlay the process of actin polymerization, dynamics, and network formation. Our integrated approach presented here allows for a direct correlation of structural data with biochemical findings and mechanical properties to systematically investigate the distinct steps involved in F-actin assembly, dynamics, and turnover. Our novel polymerization data indicate that F-actin filament formation involves additional pathways besides the commonly proposed nucleation-condensation mechanism, and they suggest a direct involvement of the so-called "lower dimer" (LD) in the polymerization process. Currently, we are investigating the structure and the exact role of the LD during actin filament assembly, dynamics, and turnover, as well as its functional significance in vivo. We have now refined our data analysis and processing methods so as to produce averaged EM-based 3-D reconstructions which, in combination with X-ray diffraction data, allow us to analyze the F-actin filament structure to atomic detail. This integrated approach represents a powerful tool to assess distinct conformational states of the actin polymer and to determine the 3-D structure of the F-actin filament in functionally important complexes with actin-binding proteins and even small effector molecules such as phalloidin. By combining the power of X-ray diffraction and/or nuclear magnetic resonance spectroscopy data of individual components at atomic resolution with EM-based 3-D reconstructions of entire supramolecular assemblies at intermediate resolution, this approach is generally applicable and plays an invaluable role in building and refining atomic models. In the case of actin, we will continue to systematically apply these methods to eventually solve the atomic structure of the F-actin filament and to gain more detailed insight into its dynamic properties and functional involvement in various cellular processes.

We thank Drs. Ron Milligan, Roger Wepf, and Mary Reedy for allowing us to use some of their EM data, and we are grateful to Dr. Cora-Ann Schoenenberger for critical reading of the manuscript. This work was supported by the Canton Basel-Stadt and the M.E. Müller Foundation of Switzerland, by a research grant from the Swiss National Science Foundation (31-39691.93), and...
by a postdoctoral fellowship awarded to A. Bremer by the Human Frontier Science Program (HFSP).

REFERENCES