An antiparallel actin dimer has been proposed to be an intermediate species during actin filament nucleation. We now show that latrunculin A, a marine natural product that inhibits actin polymerization, arrests polylsine-induced nucleation at the level of an antiparallel dimer, resulting in its accumulation. These dimers, when composed of pyrene-labeled actin subunits, give rise to a fluorescent excimer, permitting detection during polymerization in vitro. We report the crystallographic structure of the polylsine-actin-latrunculin A complex at 3.5-Å resolution. The non-crystallographic contact is consistent with a dimeric structure and confirms the antiparallel orientation of its subunits. The crystallographic contacts reveal that the mobile DNase I binding loop of one subunit of a symmetry-related antiparallel actin dimer is partially stabilized in the interface between the two subunits of a second antiparallel dimer. These results provide a potential explanation for the paradoxical nucleation of actin filaments that have exclusively parallel subunits by a dimer containing antiparallel subunits.

Actin filament nucleation occurs very slowly de novo, but it occurs rapidly as a necessary step in actin-based motility (1). The formation of a dimer from monomeric subunits is the most thermodynamically unfavorable nucleation step with an estimated equilibrium dissociation constant of 4.6 μM (in contrast to 0.6 mM for conversion of dimer to trimer) in a recent molecular simulation of nucleation (2). The formation of an effective nucleus may be accelerated in vivo by an actin-binding protein such as gelsolin, which can stabilize dimeric actin, or by a protein complex such as Arp2/3 that is thought to contain two actin-like molecules constrained in an orientation that promotes nucleation (3, 4). Antiparallel actin dimers have been identified as a precursor to actin filament polymerization by covalent cross-linking during polymerization induced with divalent cations (5). A gelsolin-actin complex capable of nucleating filament growth at the slow growing, pointed end of filaments has also been shown by covalent cross-linking to contain two actin subunits in the antiparallel configuration (6). The assumption of an antiparallel configuration of subunits is based on evidence that Cys-374 in the C terminus of actin is the only residue involved in the cross-linking reaction. In contrast, when polymerization is complete, intrafilament cross-linking yields a parallel dimer. More recently, electron microscopy has revealed that newly formed actin filaments show evidence of incorporation of antiparallel dimers. This incorporation results in a branched filament network, implying that the dimers have nucleating activity (7). Interestingly, analysis of a Listeria model of cell motility using high-resolution laser tracking provides evidence that filaments elongate in 5.4 nm steps, consistent with in vivo incorporation of dimeric actin (8).

In the current work, we provide evidence that polylsine nucleates actin polymerization by enhancing the stability of an antiparallel dimer and that the production of antiparallel dimer induced by polylsine and other nucleation-promoting agents correlates well temporally with filament nucleation. We show that the polylsine-induced dimer accumulates in the presence of latrunculin A, generating a homogeneous complex that can be crystallized. An x-ray structure of an antiparallel dimer is reported that correlates well with the available biochemical data for the solution dimer.

**EXPERIMENTAL PROCEDURES**

**Actin Polymerization and Covalent Cross-linking**—Polymerization of pyrene-labeled rabbit muscle actin (4% of actin was labeled except when detecting excimer, in which case 73% was labeled) was measured at 22 °C using a PTFI spectrophotometer with excitation at 366 nm and emission at 387 nm. Actin in buffer G (0.1 mM CaCl₂, 0.2 mM dithiothreitol, 0.2 mM ATP, 0.01% sodium azide, and 5.0 mM Tris, pH 7.8) was converted to Mg²⁺-actin by the addition of 125 μM EGTA and 50 μM MgCl₂ 15 min prior to the initiation of polymerization. For covalent cross-linking, actin, polylsine, and latrunculin A were mixed at the indicated concentrations. Polylsine (Sigma) with low polydispersity

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Pyrene-labeled actin is defined as actin labeled on Cys-374 with N-(1-pyrene)iodoacetamide.
Crystal Structure of an Antiparallel Actin Dimer

(weight-average $M_w$/number-average $M_n$ is 1.1) had an average of 24 lysine residues. Phenylendimaleimide (PDM) was dissolved in dimethylformamide at 5 mM and diluted to 10 mM sodium borate, pH 9.2, immediately prior to use. Cross-linking was initiated by the addition of equal volumes of actin and PDM to achieve a final molar ratio of either 0.51 or 4:1 PDM/actin as previously described (5) so as to optimize the yield of antiparallel or intrafilament dimer, respectively.

Analytical Ultracentrifugation—Actin, polylysine, and latrunculin A were mixed at the stated concentrations and centrifuged in a Beckman XL-A analytical ultracentrifuge at 14,400 rpm (for sedimentation equilibrium) or at 53,000 rpm (for sedimentation velocity) in buffer G plus 125 mM EGTA, 2 mM MgCl₂, and 40 mM KCl. Samples of 110 µl reached equilibrium in 36 h at 4 °C. Buffer density was 1.0015, and the partial specific volume for the complex was assumed to be that of actin alone, 0.72 ml/g (9). For sedimentation velocity, 400-µl samples were loaded into double sector cells at 20 °C. Absorbance scans were obtained at 12-min intervals at 290 nm. Sedimentation coefficients were calculated using the second-moment analysis method (10). Theoretical sedimentation coefficients were calculated using HYDROPRO (11) using the atomic coordinates of the antiparallel dimer or of a compact dimer in which two subunits interact via DNase I binding loop contacts with subdomains I and III as shown in Fig. 5A.

Results

Actin Filament Nucleation Initiated by an Antiparallel Actin Dimer—Actin filament nucleation is fast in vitro when a short oligomer of polylysine is present (Fig. 1A). The effect of polylysine is very potent relative to molecules such as phalloidin or jasplakinolide, which promote nucleation by binding at the interface of three subunits to stabilize a helical actin trimer (Fig. 1B). Given the enhanced intrinsic stability of trimer relative to dimer, an observed effect greater than that caused by a very high affinity actin-trimer ligand such as jasplakinolide is likely related to modulation of actin dimer formation (9).

Indeed, if actin is covalently cross-linked during polymerization induced by polylysine, a substantial yield of antiparallel dimers with high electrophoretic mobility on SDS-PAGE is obtained (Fig. 2A). Consistent with the cross-linking results, polylysine, at saturation, changes the slope of a log-log plot of the time required for actin to become 50% polymerized as a function of actin concentration in the presence of 5 µM polylysine or 5 µM jasplakinolide. For data satisfying nucleation-elongation kinetics, a shift to the left indicates an increase in the rate constant for nucleation, and the slope of each best fit line is equal to one-half the size of the nucleus (18).

Fig. 1. Nucleation of actin polymerization by polylysine. A, time course of 4% pyrene-labeled rabbit muscle Mg²⁺-actin polymerization with polylysine in 40 mM KCl and 2 mM MgCl₂. The final extent of polymerization is similar in all samples. B, a log-log plot of the time required for actin to become 50% polymerized as a function of actin concentration in the presence of 5 µM polylysine or 5 µM jasplakinolide. For data satisfying nucleation-elongation kinetics, a shift to the left indicates an increase in the rate constant for nucleation, and the slope of each best fit line is equal to one-half the size of the nucleus (18).

The abbreviations used are: PDM, phenylendimaleimide; MARCKS PSD, phosphorylation site domain of myristoylated alanine-rich protein kinase C substrate; r.m.s., root mean square.

2 The abbreviations used are: PDM, phenylendimaleimide; MARCKS PSD, phosphorylation site domain of myristoylated alanine-rich protein kinase C substrate; r.m.s., root mean square.
consistent with its known binding location (9).

The yield of cross-linked antiparallel dimer is substantially higher when polylysine is used to nucleate polymerization than has been previously reported for other polymer-inducing conditions (5). If cross-linking is performed as the polymerization reaction nears completion then the yield of antiparallel dimer is significantly less, and the major cross-linked product is then an intrafilament dimer with lower electrophoretic mobility. (Lane 7 of Fig. 2A shows the depletion of antiparallel dimer with time.) Both the cross-linking pattern of mature filaments and the normal appearance of negatively stained polylysine-induced filaments (20) suggest that polylysine does not alter filament structure. However, if the actin–monomer sequestering drug latrunculin A (13) is added at the initiation of polymerization, polymerization is arrested at the dimeric stage, and the antiparallel dimer accumulates (Fig. 2A, lane 6). The cross-linking reaction is not 100% efficient, and therefore monomer is expected to be visible on an SDS-denatured gel even if the dimer is homogeneous in solution. Sedimentation equilibrium experiments (performed at 4 °C as previously described (21) with a mixture of actin, polylysine, and latrunculin A, at concentrations of 5, 2.5, and 12 μM, respectively) confirm that actin is homogeneously dimeric (Fig. 2B), even in the presence of high concentrations of divalent cation (2 mM MgCl2). Results of sedimentation velocity experiments (data not shown) performed under identical conditions (except at a temperature of 20 °C) also reveal a homogeneous species with sedimentation coefficient ($s_{20, w}$) of $4.81 ± 0.10$ S. Because these results show that the actin is dimeric in solution rather than filamentous, the antiparallel cross-link cannot be the result of interfilament cross-linking in a bundle of filaments, as has been speculated to occur in other conditions (7). Cross-linking and sedimentation equilibrium experiments in the presence of excess latrunculin A showed no evidence of dimer formation in the absence of polylysine.

The finding that an antiparallel dimer was an intermediate species during polylysine-induced actin polymerization was unexpected. Given the parallel orientation of subunits in an actin filament, the antiparallel geometry is paradoxical, with a requisite large change in subunit orientation if both subunits are incorporated into a filament. Moreover, in general, conditions that provoke rapid nucleation, e.g., 2 mM MgCl2, have previously been associated with relatively lower yields of antiparallel dimer (5). Indeed, because of these considerations, it has been speculated that the antiparallel dimer could be an inert product of a bimolecular reaction mechanism that is independent of actin polymerization (7). The evidence that ubiquitous antiparallel dimer formation correlates well temporally with nucleation events and the evidence of incorporation during filament polymerization argue that this is not the case (6, 7). Our new data for polylysine are particularly convincing in this regard, as the apparent abundance of antiparallel dimer during extremely rapid polymerization is difficult to reconcile with the idea that the dimer represents actin diverted to an unpolymerizable state.

For more than 20 years since the discovery of the antiparallel actin dimer (5, 22) there has been no technique to allow for its detection other than covalent cross-linking, a procedure requiring extended incubation times and high pH levels (5). We now find evidence that pyrene-labeled actin forms an excimer in the antiparallel dimer (and not in F- or G-actin), providing a “real-time” assay for monitoring its formation (Fig. 3A). Because the presence of excimer implies that the pyrene fluorophores are less than 18 Å apart (23), this is consistent with the biochemical evidence of antiparallel dimer. Of course, this is not proof that antiparallel dimer is present whenever excimer is detected, nor proof of the converse. However, the strong correlation between dimer formation and conditions/times yielding cross-linking of antiparallel dimer suggests that these conclusions are reasonable. Polylysine induces rapid formation of excimer, which decreases in abundance with time (Fig. 3B). The less potent polyacatonic nucleating factor, the phosphorylation site domain of myristoylated alanine-rich protein kinase C substrate (MARCKS PSD), causes accumulation of excimer at a slower rate followed by depletion during polymerization (Fig. 3B). Polymerization of Ca2+-actin is accompanied by the formation of a small amount of excimer during the time interval that corresponds to filament nucleation (Fig. 3C), providing the first corroborating evidence of antiparallel dimer formation during in vitro polymerization of Ca2+-actin.

Crystal Structure of an Antiparallel Actin Dimer—Dimer homogeneity in the presence of latrunculin A unexpectedly implied that polylysine, even though it strongly promoted two-dimensional polymerization, could be exploited to make crys-
The surface area of 2.5

volves the DNase I binding loop, with a buried interfacial

domain indicative of oligomerization. These other structures have no demonstrable non-crystal-

polylysine that prevents polymerization (24, 25, 26), or more recently, a

have required the presence of an actin-binding protein

biochemical data shown in Fig. 2, the structure reported here is

complex of profilin and

covalently modified form of ADP-actin that is polymerization

unlike that recently described for the crystal structure of a

subunit of one dimer is sandwiched within a pocket created by

interaction confers stability to only one loop; the other one is

linking reagent such as PDM (12). The DNase I binding loop of one

DNase I binding loop, but this is unlikely

to be the precursor complex that adds to the barbed end of
growing filaments (30). In light of a previous report that <0.1

kcal/mol might be required for the transition between the two

most extreme positions of subdomain 2 shown in Fig. 6C, the

full range of depicted conformations are likely represented in

solution (30). Our results, therefore, suggest that the DNase I

binding loop is likely molded into the observed conformation by

its interaction with an antiparallel actin dimer. Alternatively,
dimerization, like many other binding events at the C terminus

of actin (31), may have induced allosteric changes in the con-

formation of the DNase I binding loop, but this is unlikely

because both DNase I binding loops are not similarly struc-
tured in this otherwise symmetrical antiparallel dimer.

**DISCUSSION**

Nucleation of actin polymerization by antiparallel dimer as

reported here has been previously observed qualitatively (7).
The relationship between the crystallographic and solution

structures of antiparallel actin dimer is of interest. The disul-

fide bond that we observe in the crystallographic structure very

likely does not form until after crystallization is complete, given

that the PDM cross-linking and analytical ultracentrifug-

data prove definitively that the dimer exists in solution

without a disulfide (the PDM reaction requires a reduced cys-
teine). The flexibility of the C terminus reported by others may

account for the observation that a finite length, covalent cross-

linking reagent such as PDM (~12 Å separates the reactive

maleimide groups) can cross-link antiparallel dimeric actin in

solution, whereas the cysteine residues are fixed in space at a

distance of 6.8 Å (Ca to Co) by the disulfide bond in the crystal

structure. Similarly, the ability to form a pyrene excimer im-

plies that the antiparallel dimer can accommodate a bulky

fluorescent probe at the subunit interface. The two salt bridges

found in the dimer interface provide a thermodynamic ration-
al for the stability of the dimer in solution in the absence of
disulfide, and hydrodynamic data support the contention that

the crystal and solution dimers are of similar shape. The ex-

pected sedimentation coefficient of the antiparallel actin dimer
can be estimated directly from the atomic coordinates (11),
yielding a value of 4.85 S, a result that compares very well with the experimental value of 4.81 ± 0.10 S. In contrast to this end-to-end dimer, a compact side-to-side actin dimer yields a sedimentation coefficient of 5.25 S by the same methodology. Dynamic properties of the antiparallel dimer in solution remain unexamined and add potential complexity to this analysis. In summary, the crystallographic dimer is probably not identical to that occurring in solution (and, in fact, the flexible C-terminal residues may not have a well defined structure in solution), but the evidence strongly supports the following: 1) that both crystallographic and solution structures are dimeric, 2) that the dimer interface in the crystal is similar to that present in solution, and 3) that the sedimentation coefficient is reasonable for a dimer of the shape predicted by the crystal structure.

When latrunculin A was soaked into gelsolin-actin crystals only minor changes were observed in actin structures involving loops between residues 55–66 and 197–207 in subdomains II and IV, respectively (15). Because of the absence of identifiable alterations that fully explain the inability of actin to polymerize. In contrast to this end-to-end dimer, a compact side-to-side actin dimer yields a sedimentation coefficient of 5.25 S by the same methodology. Dynamic properties of the antiparallel dimer in solution remain unexamined and add potential complexity to this analysis. In summary, the crystallographic dimer is probably not identical to that occurring in solution (and, in fact, the flexible C-terminal residues may not have a well defined structure in solution), but the evidence strongly supports the following: 1) that both crystallographic and solution structures are dimeric, 2) that the dimer interface in the crystal is similar to that present in solution, and 3) that the sedimentation coefficient is reasonable for a dimer of the shape predicted by the crystal structure. In summary, the crystallographic dimer is probably not identical to that occurring in solution (and, in fact, the flexible C-terminal residues may not have a well defined structure in solution), but the evidence strongly supports the following: 1) that both crystallographic and solution structures are dimeric, 2) that the dimer interface in the crystal is similar to that present in solution, and 3) that the sedimentation coefficient is reasonable for a dimer of the shape predicted by the crystal structure. In summary, the crystallographic dimer is probably not identical to that occurring in solution (and, in fact, the flexible C-terminal residues may not have a well defined structure in solution), but the evidence strongly supports the following: 1) that both crystallographic and solution structures are dimeric, 2) that the dimer interface in the crystal is similar to that present in solution, and 3) that the sedimentation coefficient is reasonable for a dimer of the shape predicted by the crystal structure. In summary, the crystallographic dimer is probably not identical to that occurring in solution (and, in fact, the flexible C-terminal residues may not have a well defined structure in solution), but the evidence strongly supports the following: 1) that both crystallographic and solution structures are dimeric, 2) that the dimer interface in the crystal is similar to that present in solution, and 3) that the sedimentation coefficient is reasonable for a dimer of the shape predicted by the crystal structure.
The crystallographic contact reveals a large surface of interaction between the DNase I binding loop of subdomain 2 and subdomains 1 and 3 of another subunit (subdomain nomenclature is defined in Fig. 4B). This interaction involves the same surface as the site of longitudinal interaction between subdomain 3 and the DNase I binding loop that occurs along the long pitch axis of F-actin in the Lorenz model of the filament, a model based on low resolution diffraction data from F-actin gels (16, 32). Interestingly, the extended loop in our crystal structure reveals a much more extensive surface of interaction than that of the filament model (32). This is significant because a structure derived from crystals of profilin-actin contains actin-actin contacts of the DNase I binding loop with subdomain 4 and with the N terminus, and both contacts are distinct from those of the Lorenz model. The large surface area of the contacts in the profilin-actin crystal compared with those of the Lorenz model has led to controversy regarding the model’s validity (26). Normal mode refinements in F-actin structure support the conclusion that the DNase I binding loop cannot be uniquely oriented given available data (29), but similar to the crystallographic contact reported here, the refined filament structure includes long pitch axis subunit contacts with subdomain 1 at residues Ser-350 and Thr-351 (Fig. 6B). These results suggest that specific features of the crystallographic contact might be incorporated into the current model of F-actin without significant repositioning of the DNase I loop, resulting in more extensive long pitch helical contact and lower global free energy.

The crystallographic contact between the DNase I binding loop and subdomains 1 and 3 provides the basis for a hypothesis that explains the paradox regarding participation of an antiparallel actin dimer in the helical polymerization of parallel subunits. The extensive surface of interaction and hydrophobic burial suggests that this contact could reflect an authentic solution interaction. Because of stabilization of the mobile DNase I binding loop of a third actin subunit at the interface between the two subunits of the dimer, the association of a monomer with antiparallel dimer in this configuration is expected to be more favorable than the association of two parallel monomers, assuming otherwise similar binding surfaces. The energy. The crystallographic contact between the DNase I binding loop of a third actin subunit at the interface between the two subunits of the dimer, the association of a monomer with antiparallel dimer in this configuration is expected to be more favorable than the association of two parallel monomers, assuming otherwise similar binding surfaces. The stability conferred to the DNase I binding loop by its sand-
permit extensive realignment. Rotation of the DNase I loop-bound subunit by ~90° along the longitudinal axis of the dimer is required to bring two subunits into an orientation consistent with models of F-actin (Fig. 7). This mechanism does not require that the crystallographic contact between the DNase I loop and subdomains 3 and 1 be identical to that occurring in F-actin. The flexibility of the DNase I loop may be sufficient so that any stable tethering of the loop of one subunit to the antiparallel dimer would increase the effective concentration of reactants and augment the association rate constant.

Experimental data consistent with a nucleus size of 2–5 actin subunits have been reported by others (19, 33, 34). Part of this discrepancy can be explained by differences in the definition of the nucleus (35, 36). A recent theoretical thermodynamic analysis by Sept and McCanmon (2) did not consider possible conformation transitions between oligomeric actin structures. In the absence of this consideration, the effective nucleus size is defined by assumptions implicit in the structural model of the nucleus, and more specifically, by the number of different types of actin-actin contacts in the helical oligomer. Given a model (2) employing two different possible actin-actin intrafilament contacts, the addition of a fourth subunit to a trimer would represent the same free energy change as that associated with the addition of another subunit to a long filament (except for very small differences in change in entropy as estimated in Ref. 37). Theoretical log-log plots of actin polymerization (as in Fig. 1B) based on this model would yield a slope of 2, consistent with the experimental data (2, 36). However, conformational transitions were introduced by Oosawa and Asakura (18) as alternative pathways for nucleation and elongation at the barbed end of a filament following nucleation by an antiparallel dimer. As previously observed in samples viewed by electron microscopy (7), one subunit of the antiparallel dimer is not incorporated into the filament.

There are several questions associated with the model in Fig. 7. However, it should be remembered that no alternative explanation for paradoxical nucleation by antiparallel dimer has been proposed despite more than 20 years of investigation (22). Most notably, actin-actin crystallographic contacts may not be predictive of solution interactions. There is at present no evidence that the DNase I loop binds to antiparallel dimer in solution as seen in the crystallographic contact. More generally, there is also no evidence that a third subunit binds to solution to the dimer, with Fig. 2B showing that there is no self-association of dimers at concentrations of up to ~10 μM. However, it should be noted that even if the equilibrium dissociation constant was in the range of 1–3 mM, this mechanism would still be 1000-fold more effective at creating a linear dimer than through an intrafilament dimer mechanism (2). There is also the concern that limitations in the range of mobility of the DNase I binding loop could prevent the conformational reorganization depicted in Fig. 7B, but this is tempered by the absence of proof that the solution structure of an actin filament is identical to any of the proposed filament models.

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