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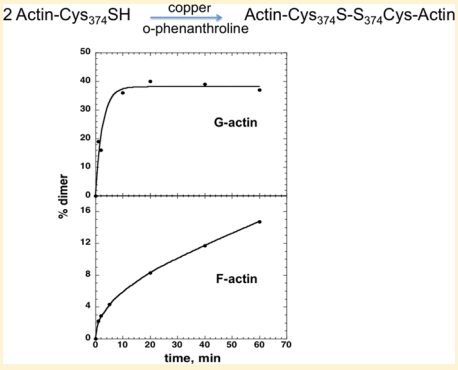
Disulfide Cross-Linked Antiparallel Actin Dimer

Philip Graceffa,* Eunhee Lee, and Walter F. Stafford

Boston Biomedical Research Institute, Watertown, Massachusetts 02472, United States

ABSTRACT: Oxidation of actin monomer (G-actin) with copper *o*-phenanthroline resulted in a rapid, high yield of disulfide cross-linked dimer. The cross-link is due to an intermolecular disulfide bond between actin Cys374 of each molecule, resulting in a tail-to-tail, i.e., antiparallel, actin dimer. Analytical ultracentrifugation profiles of G-actin can be ascribed to the existence of actin monomers with very little, if any, dimer. Thus, actin dimers are not energetically favorable, indicating that cross-linked dimers are formed during random diffusional collisions. On the other hand, a similar oxidation of actin polymer (F-actin) resulted in a much lower yield of the cross-linked actin dimer that showed no sign of leveling off. Therefore, it is proposed that the cross-linked dimer from actin polymer is due to collisional complexes of actin monomers that are in equilibrium with the polymer during actin treadmilling. These results account for the reported observation that during the early stages of actin polymerization (where the actin monomer concentration is high) cross-linked antiparallel actin dimers are formed in relatively high yield whereas none are formed at later stages of polymerization. These findings raise questions concerning the validity of the antiparallel actin dimer model of in vitro actin polymerization that is based on the assumption that the ability to form cross-linked actin dimers implies the existence of stable dimers.



The actin filament is composed of actin monomers bound head to tail, i.e., parallel binding, that form two intertwining helical strands. The actin filament is a dynamic structure by virtue of its ability to accommodate a continuous net addition of ATP−actin monomers to the barbed end of the filament and a net dissociation of ADP−actin monomers from the pointed end, a process termed treadmilling and fueled by ATP hydrolysis.[1] This dynamic is the basis of the capacity of cells to change shape and to be motile and is controlled by actin binding proteins. In vitro, polymerization of actin to filaments (filamentous or F-actin) is initiated by the addition of salt to low-ionic strength solutions of actin monomers (globular or G-actin). Its progress is followed by the increase in either the light scattering or the fluorescence of covalently attached probes. Actin polymerization takes place in two phases, a slow nucleation phase and a more rapid elongation phase.

In vitro actin polymerization appears to pass through different stages of polymer formation. It has been observed by electron microscopy that during the initial stages of polymerization the "young" actin filaments are ragged, branched, unstable, and irregular, whereas at later stages the "mature" filaments are longer, straighter, stable, and smooth.[2−7] In some cases, the mature filaments can revert to filaments that resemble young filaments.[5,6] Another study has proposed that actin polymerization involves a transition through several actin filament states based on the fluorescence of a probe attached to actin.[8]

There are several models that have been put forth to explain this transition between actin filament types. One model holds that, early in polymerization, the filament incorporates antiparallel actin dimers, which disrupt the smooth addition of subsequent actin monomers.[2−4,7,9,10] This model further stipulates that, with time, these antiparallel dimers revert to the normal parallel orientation, which results in the mature filament structure. A second model proposes that actin filaments have an inherent reversible structural plasticity, which allows actin filaments to assume either a young or a mature structural state depending on the conditions.[5,6] It has been proposed that this plasticity might be due to the observation that actin monomers assume different tilted orientations within the filament.[6,11] Finally, a third model states that actin filaments irreversibly pass through several well-defined filament conformations.[8] The latter two models do not invoke the existence of antiparallel actin dimers.[5,6,8,11]

The basis of the antiparallel actin dimer model is the observation that in the early stages of polymerization one is able to form cross-linked antiparallel actin dimers by cross-linking actin Cys374 of one molecule to actin Cys374 of another via divalent chemical cross-linkers.[2−4,7,9,10] In later stages of polymerization, during the formation of mature filaments, the extent of cross-linked antiparallel dimer formation diminishes greatly. This model assumes that the ability to form cross-linked actin dimers implies the existence of stable actin dimers in solution. This work was initiated to test this assumption by using the technique of chemical cross-linking coupled to that of analytical ultracentrifugation. This investigation provides evidence that indicates that this

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assumption is not true, which calls into question the validity of the antiparallel actin dimer model of actin polymerization.

MATERIALS AND METHODS

Actin Preparation. Rabbit skeletal muscle actin was prepared as described previously.[12] Some actin was modified with NEM[13] or digested with *Staphylococcus aureus* V8 protease[14] as reported previously. Actin was labeled at Cys374 with tetramethylrhodamine-5-maleimide (Invitrogen) as described previously.[15,16] Actin was generally stored for up to ~2 weeks on ice as monomers in low-ionic strength G-buffer [2 mM Mops, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.01% NaN_3 (pH 7.5)]. The actin concentration was determined from the optical density at 290 nm minus that at 320 nm using an extinction coefficient $A^{1\%}_{290}$ of 6.3 cm^{-1}.[17] Actin was polymerized via addition to actin (in G-buffer) of NaCl to 40 mM and $MgCl_2$ to 2 mM (F-buffer).

Sulfhydryl Content. The total sulfhydryl content of actin denatured in 4 M guanidine hydrochloride was determined colorimetrically by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma).[18]

Oxidation by Copper Phenanthroline. A stock solution of 5 mM Cu(*o*-phen)$_2$ was prepared by dissolving *o*-phenanthroline (Fisher Scientific) to a final concentration of 10 mM in 5 mM $CuSO_4$. The reaction between Cu(*o*-phen)$_2$ and actin was initiated by adding 1 mM Cu(*o*-phen)$_2$ to actin to the appropriate final concentration, and the reaction was stopped by adding excess (2−5 mM) NEM to block actin sulfhydryls for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Carboxypeptidase Treatment. Actin was treated with carboxypeptidase A (CP-A) (Sigma) essentially as described previously where up to three C-terminal residues (Phe375, Cys374, and Lys373) were removed.[19] CP-A was added to actin monomer in G-buffer at a CP-A:actin molar ratio between 0 and 0.04 and reacted for 0.5−3 h at room temperature. At the end of the reaction time, Cu(*o*-phen)$_2$ was added at a Cu(*o*-phen)$_2$:actin molar ratio of 1.0 and reacted for 0.5 h at room temperature. This reaction was terminated with 5 mM NEM, whereupon SDS−PAGE was conducted.

Gel Filtration Chromatography. Gel filtration chromatography of actin or Cu(*o*-phen)$_2$-treated actin was conducted on a 16 mm × 60 cm Pharmacia Superdex 200 column. Before application to the column, the treated actin was dialyzed against G-buffer to remove the Cu(*o*-phen)$_2$. The actin was applied to the column in G-buffer or G-buffer containing 0.3 M NH_4SCN and eluted with the same buffer. Column fractions were monitored by the optical density at 290 nm, and aliquots were treated with 2 mM NEM to block all free sulfhydryl groups before SDS−PAGE. Monomer and dimer fractions were pooled and concentrated by dialysis versus 10% PEG 20K in G-buffer and then versus G-buffer. In some cases, the separated monomer and dimer were treated overnight on ice with 10 mM DTT to reduce disulfide bonds and then dialyzed against G-buffer to remove DTT.

Peptide Sequence Analysis. Actin peptide sequence analysis was performed by Edman degradation on an Applied Biosystems sequencer (model 477A) with an online phenylthiohydantion analyzer (model 120A).

SDS−PAGE. SDS−PAGE was performed at 10% acrylamide according to Laemmli.[20] Gels were scanned with an Epson Expression 1680 scanner, and densitometry of the scans was performed with the NIH ImageJ program.

Analytical Ultracentrifugation. Analytical ultracentrifugation sedimentation velocity experiments were conducted and the results analyzed essentially as detailed previously.[21] SEDANAL[22] was used to calculate the sedimentation velocity weight-average sedimentation coefficients; SEDNTERP[23] was used to estimate hydration and to convert sedimentation coefficients to the standard condition of 20 °C in water, $s_{20,w}$, and SEDANAL[22] was used to test sedimentation velocity data for interacting models.

Actin Polymerization. Polymerization of actin or ASSA (both in G-buffer with or without 12.5 mM DTT) in the presence of 2−5% TMR-actin was initiated by the addition of 1.1%, by volume, of a concentrated NaCl/$MgCl_2$ solution such that the final concentrations were 40 mM NaCl and 2 mM $MgCl_2$. The final concentration of actin (monomer equivalent) was 2 μM. Samples were incubated for 1 h at 20 °C before initiation of polymerization to ensure complete reduction of the ASSA disulfide bond by DTT. Polymerization was monitored by the increase in the 575 nm fluorescence of TMR-actin excited at 530 nm. Fluorescence was measured on a Varian Eclipse spectrofluorometer equipped with a Peltier temperature regulator that was set at 20 °C. Although TMR-actin alone is polymerization incompetent,[15] it copolymerizes with unlabeled actin[24] with an approximately 75% increase in TMR fluorescence (see Figure 8).

RESULTS

G-Actin Monomers. *Copper o-Phenanthroline Oxidation.* Cu(*o*-phen)$_2$ has previously been reported to oxidize proteins via disulfide bond formation.[25−28] Cu(*o*-phen)$_2$ treatment of actin at a molar ratio of 1:1 resulted in the generation of an additional SDS−PAGE band that migrated with a molecular mass of 85 kDa (Figure 1A), roughly twice

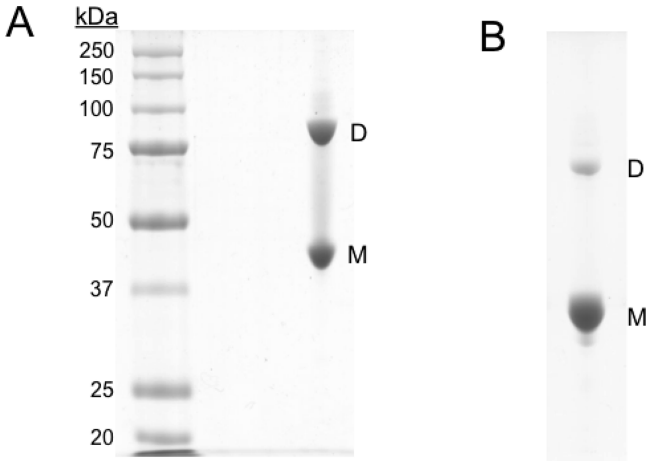

Figure 1. SDS−PAGE of Cu(*o*-phen)$_2$-oxidized G-actin. (A) Molecular mass standards and 100 μM actin treated with equimolar Cu(*o*-phen)$_2$ for 1 h at room temperature in G-buffer. (B) NEM-actin (83 μM) treated with equimolar Cu(*o*-phen)$_2$ for 1 h at room temperature in G-buffer. D stands for dimer and M for monomer.

that of the actin monomer (42 kDa). Treatment of the Cu(*o*-phen)$_2$-oxidized actin with DTT, a reagent that reverses disulfide bond formation by disulfide exchange,[29] resulted in the loss of the dimer band (not shown). Therefore, the 85 kDa band is a disulfide cross-linked actin dimer.

We can conclude that the actin dimer bond is a disulfide bond between Cys374 of one molecule and Cys374 of another,

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i.e., a tail-to-tail or antiparallel dimer, for the following four reasons. (1) Cu(*o*-phen)$_2$ treatment of actin modified with NEM, which specifically blocks Cys374,[30,31] showed an actin dimer band on SDS−PAGE with a greatly diminished magnitude (Figure 1B). (2) CP-A-treated actin, which has Phe375, Cys374, and Lys373 removed,[19] also resulted in a reduction in the level of Cu(*o*-phen)$_2$-generated dimer formation (Figure 2). The size of the reduction increased

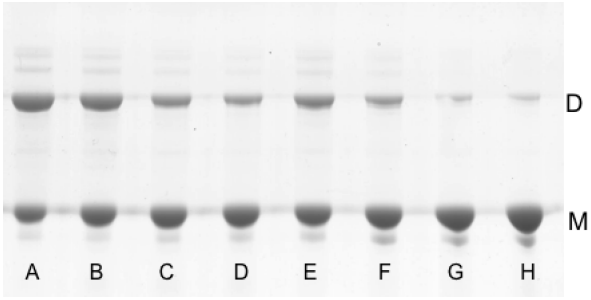

Figure 2. SDS−PAGE of Cu(*o*-phen)$_2$-oxidized, carboxypeptidase A-treated G-actin. CP-A-treated actin at 153 μM was reacted with equimolar Cu(*o*-phen)$_2$ for 30 min at room temperature in G-buffer: lane A, control actin; lanes B−D, actin treated with CP-A for 1 h at room temperature at actin:CP-A molar ratios of 100, 50, and 25, respectively; lanes E−H, actin treated with CP-A at an actin:CP-A molar ratio of 25 for 0.5, 1, 2, and 3 h, respectively. D stands for dimer and M for monomer.

with increasing CP-A reaction time and with an increase in the CP-A:actin molar ratio at a fixed reaction time (Figure 2). (3) The apparent molecular mass of 85 kDa for the dimer is the same as that of the antiparallel cross-linked actin dimer formed by reaction, via Cys374, with a bifunctional sulfhydryl cross-linking reagent.[9] On the other hand, a parallel cross-linked actin dimer, formed by the same bifunctional reagent, migrated on SDS−PAGE with a mass of 115 kDa.[9] (4) Finally, digestion of the actin dimer with V8 protease resulted in a peptide that contained a cystine residue containing two Cys374 residues, as determined by Edman degradation sequencing (not shown). A similar disulfide cross-linked antiparallel actin dimer has been observed after freezing and thawing of actin[32] or of disulfide-modified actin.[33]

The maximal yield of the disulfide cross-linked actin dimer, formed under the low-salt G-actin buffer conditions, was always close to 50% (Figure 1A), ranging from 40 to 54%. This maximal yield was achieved with a Cu(*o*-phen)$_2$:actin molar ratio of 1.0 and was not further increased with increasing Cu(*o*-phen)$_2$:actin molar ratios of up to 5.0 (Figure 3). Increasing the ratio beyond 5.0 did increase the dimer yield somewhat, but an actin precipitate also formed, probably because of Cu^{2+}-induced aggregation. The maximal yield was reached in approximately 10 min (Figure 4A). The dimer yield was relatively insensitive to pH values from 7.0 to 8.0 and to temperatures from 0 to 25 °C. The reaction yield did increase with actin concentration, reaching 50% dimer at ∼100 μM actin (Figure 5).

It was not convenient to study the ionic strength dependence of the actin dimerization reaction in the monomeric state because the addition of salts polymerizes actin. Therefore, we examined the reaction at low ionic strengths, i.e., 0−10 mM NaCl, where actin does not polymerize. The dimerization yield was insensitive to ionic strength and remained at 45% over this range. Another approach was to use relatively high ionic strengths, which is known to reversibly depolymerize F-actin.[34]

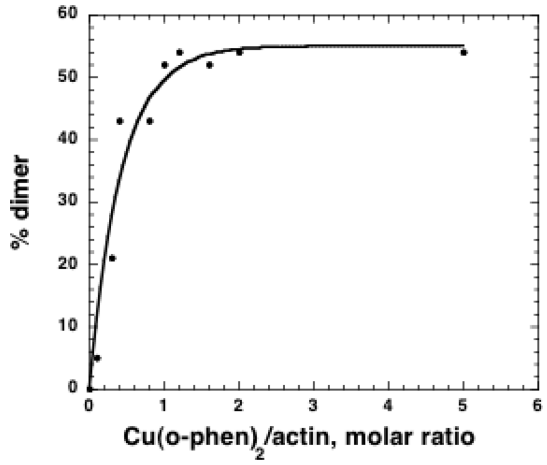

Figure 3. G-Actin dimer formation as a function of Cu(*o*-phen)$_2$:actin molar ratio. Percentage of actin as a dimer after reaction of 25 μM actin with Cu(*o*-phen)$_2$ at room temperature for 10 min in G-buffer. Dimer quantified by densitometry of SDS−PAGE.

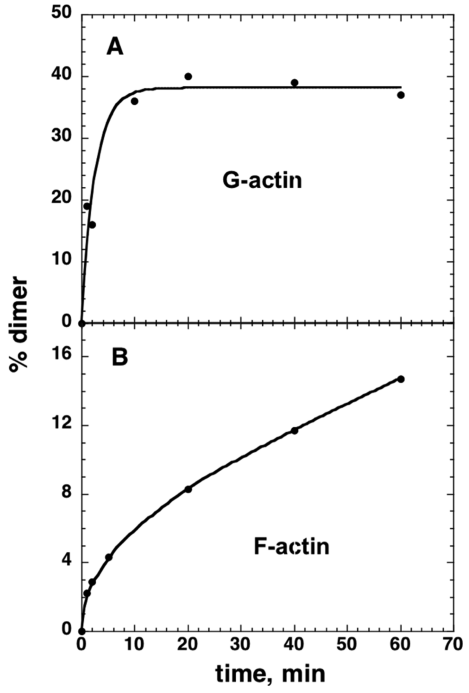

Figure 4. Time dependence of actin dimer formation upon Cu(*o*-phen)$_2$ oxidation. Percentage of actin as a dimer after reaction of 25 μM actin with equimolar Cu(*o*-phen)$_2$ at room temperature in G-buffer (A) or F-buffer (B). In panel B, actin was polymerized in F-buffer for 45 min at room temperature before Cu(*o*-phen)$_2$ was added. Dimer quantified by densitometry of SDS−PAGE.

From this previous work,[34] we speculated that NH_4SCN might efficiently depolymerize actin. Indeed, at a NH_4SCN concentration of 0.3 M in G-buffer, an ionic strength (with NaCl or KCl) generally resulting in actin polymerization, almost all of the actin was monomeric (Figure 9B). Reaction of actin with Cu(*o*-phen)$_2$ as a function of NH_4SCN concentration resulted in a dimer yield ranging from 50% at 0 M NH_4SCN to 51% at 0.1 M NH_4SCN to 64% at 0.3 M NH_4SCN to 68% at 0.4 M NH_4SCN. Thus, the level of cross-linked dimer formation increases moderately with an increasing ionic strength.

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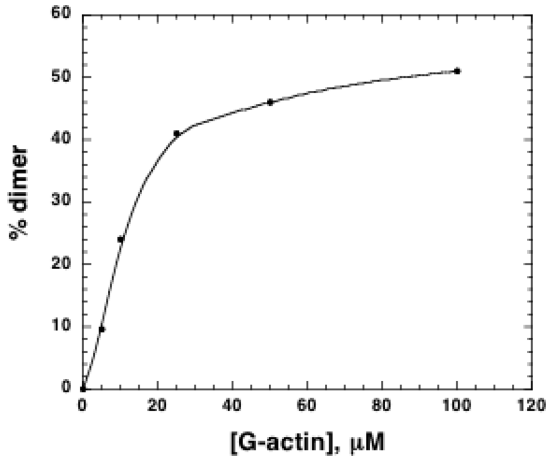

Figure 5. G-Actin concentration dependence of actin dimer formation upon oxidation with Cu(*o*-phen)$_2$. Percentage of actin as a dimer after reaction of actin with equimolar Cu(*o*-phen)$_2$ for 10 min at room temperature in G-buffer. Dimer quantified by densitometry of SDS–PAGE.

It is curious why the level of cross-linked actin dimer formation is limited to ~50% in G-buffer. One possibility is that there are two populations of actin, one that can form cross-linked dimer and one that cannot. To test this, we separated Cu(*o*-phen)$_2$-oxidized (in G-buffer) actin into the dimer and monomer by gel filtration chromatography. Chromatography in G-buffer resulted in only partial separation, whereas full separation was afforded by chromatography in G-buffer containing 0.3 M NH_4SCN (Figure 6). This suggests that

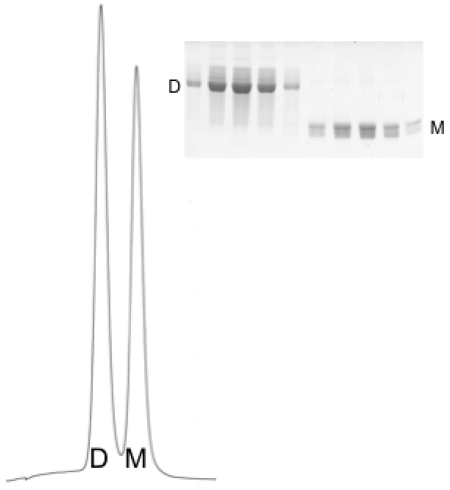

Figure 6. Gel chromatography of Cu(*o*-phen)$_2$-oxidized G-actin. Superdex 200 gel chromatography in G-buffer with 0.3 M NH_4SCN. D stands for dimer and M for monomer. The inset shows SDS–PAGE of column fraction aliquots.

there is an interaction between actin and the column matrix in the low-ionic strength G-buffer. The separated monomer and dimer were treated with DTT to reduce disulfide bonds, dialyzed to remove DTT, and assayed for total sulfhydryl content and also re-reacted with Cu(*o*-phen)$_2$. If the hypothesis described above is correct, then the DTT-reduced dimer should form close to 100% cross-linked dimer and the monomer should form none. Upon reaction with Cu(*o*-phen)$_2$, the reduced monomer formed no dimer whereas the reduced dimer formed close to 50% dimer (Figure 7), the same as the untreated actin (Figure 7). This indicated that actin was not a

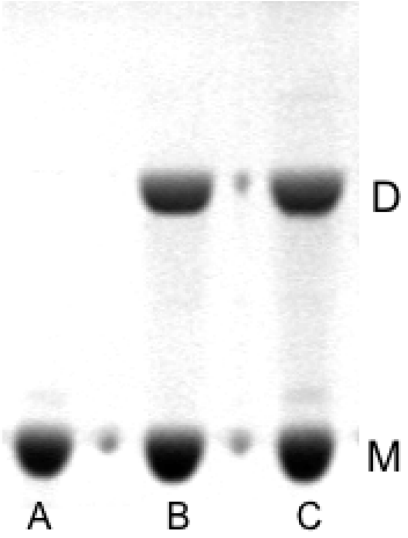

Figure 7. Reoxidation by Cu(*o*-phen)$_2$ of purified and reduced actin monomer and dimer. Cu(*o*-phen)$_2$-oxidized (for 1 h) G-actin separated into the monomer and dimer was followed by DTT reduction and reoxidation by Cu(*o*-phen)$_2$: (A) monomer, (B) dimer, and (C) actin control. Reoxidation was of 67 μM G-actin with equimolar Cu(*o*-phen)$_2$ for 15 min on ice in G-buffer. M stands for the monomer band and D for the dimer band.

heterogeneous mixture of two actin molecules but that Cu(*o*-phen)$_2$ was reacting with actin Cys374 to form an intermolecular disulfide bond or oxidizing Cys374 to a DTT-irreversible oxidation state. Indeed, the sulfhydryl content of the dimer and monomer confirmed this conclusion and further demonstrated that Cu(*o*-phen)$_2$, in addition, modified a second sulfhydryl group in the actin monomer (Table 1). That is, the

Table 1. SH:Actin Molar Ratio in 4 M Guanidine Hydrochloride[a]

	A	B
unoxidized actin	4.8	4.8
M	3.0	3.45
M + DTT	2.75	3.7
D	3.9	3.75
D + DTT	4.8	4.8

[a]Oxidation reaction in G-buffer (column A) or G-buffer with 0.3 M NH_4SCN (column B). M stands for monomer and D for dimer separated from Cu(*o*-phen)$_2$-oxidized (for 1 h) actin by gel chromatography. M + DTT and D + DTT stand for the monomer and dimer, respectively, reduced with DTT followed by DTT removal by dialysis.

monomer lost two sulfhydryls of a total of five for actin, which were not recovered by DTT treatment. The dimer lost only one sulfhydryl per monomer component, which was recovered by DTT treatment. The level of Cu(*o*-phen)$_2$ oxidation of actin sulfhydryls to a DTT-insensitive oxidation state was lower when Cu(*o*-phen)$_2$ treatment was conducted in the presence of NH_4SCN than in its absence (Table 1). This most likely contributes to the greater yield of disulfide cross-linked dimer in the presence of 0.3 M NH_4SCN.

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The polymerization capacity of the disulfide cross-linked dimer (ASSA) is greatly diminished compared to that of control actin but is fully recovered upon reduction of the disulfide bond (Figure 8). Both control actin and ASSA were purified by gel chromatography in 0.3 M NH_4SCN. The polymerization profile of actin purified in the presence or absence of NH_4SCN was identical (not shown), indicating no irreversible deleterious effect of NH_4SCN on actin polymerization. These results demonstrate that reduced ASSA is fully polymerization competent, which is consistent with the results described above (Table 1) that show that for ASSA only the cysteine involved in the disulfide bond is modified by the Cu(*o*-phen)$_2$ treatment.

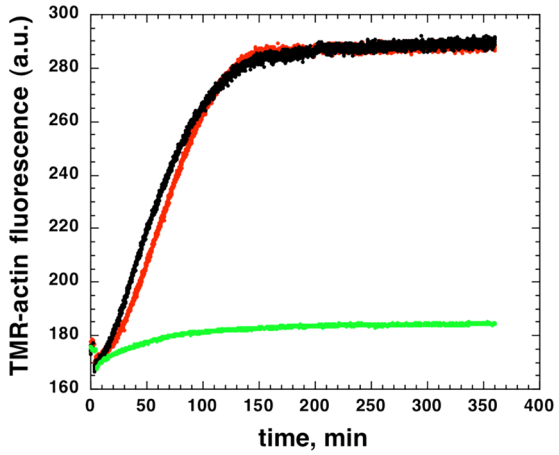

Figure 8. Polymerization of actin, ASSA, and reduced ASSA at 2 μM actin (monomer equivalent). Polymerization vs time at 20 °C in G-buffer containing 40 mM NaCl and 2 mM $MgCl_2$ with or without 12.5 mM DTT was monitored by the 575 nm fluorescence of 5% of the TMR-actin reporter molecule: actin with DTT (red), ASSA (green), and ASSA with DTT (black).

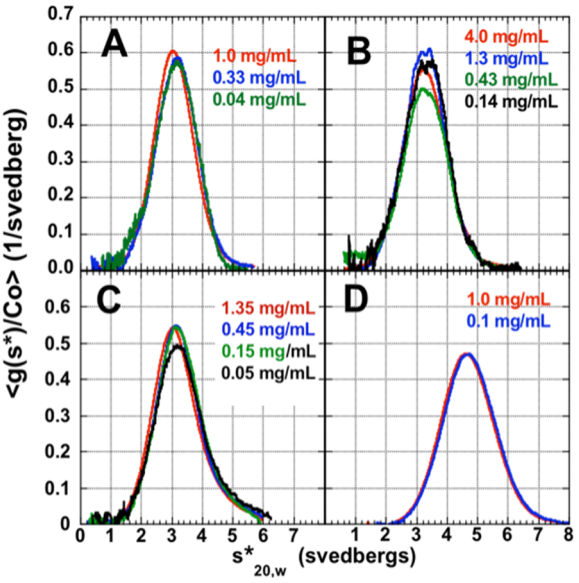

Figure 9. Analytical ultracentrifugation sedimentation velocity of actin: (A) actin in G-buffer with 5 mM DTT, (B) actin in G-buffer with 0.3 M NH_4SCN and 5 mM DTT, (C) NEM-actin in G-buffer and Cu(*o*-phen)$_2$ equimolar to NEM-actin, and (D) purified cross-linked antiparallel actin dimer in G-buffer. The concentration-normalized sedimentation distribution function (*y*-axis) is plotted vs the apparent sedimentation coefficient (*x*-axis). All runs were conducted at 20 °C.

Analytical Ultracentrifugation. The question of whether actin, under the conditions that result in a high yield of cross-linked dimer, forms a stable dimeric species arises, and if so how much. Cross-linking cannot reveal this information because it shifts the equilibrium between the monomer and dimer toward the dimer. However, analytical ultracentrifugation (AUC) can measure the equilibrium distribution between monomer and higher-order species without any significant shift in the equilibrium.

AUC sedimentation velocity of actin in G-buffer resulted in a profile that was best fit with a monodisperse species with a molecular mass of 45 kDa and a sedimentation coefficient of 3.2 S over a range of concentrations at which high yields of cross-linked dimers were formed (Figure 9A). Curve fitting was conducted using a nonideal, single-component model. Attempts to fit to a nonideal monomer−dimer model returned a value of 0.0 for the dimerization constant. Very similar results were obtained for actin in 0.3 M NH_4SCN (Figure 9B) in which even higher yields of cross-linked dimer were obtained. In both cases, the actin had a sedimentation coefficient close to 3.2−3.25 S, indicating that there were no gross shape changes in the actin molecule upon going from 0 to 0.3 M NH_4SCN. In 0.3 M NH_4SCN, there was a small amount of material (<1% of the actin) sedimenting at an apparent sedimentation coefficient of >10 that most likely corresponds to actin oligomers or aggregates.

We wished to test the possibility that Cu(*o*-phen)$_2$ was inducing the formation of a stable complex in which the two phenanthroline moieties of Cu(*o*-phen)$_2$ bridged two actin monomers whereupon the monomers would be oxidized to an intermolecular disulfide dimer. Therefore, we measured the sedimentation velocity of NEM-modified actin, which does not become oxidized by Cu(*o*-phen)$_2$ to a dimer (Figure 1B), in the presence of equimolar Cu(*o*-phen)$_2$. The resulting velocity data were best fit by an actin monomer with a sedimentation coefficient close to 3.2 with a small amount (5%) of actin dimer that did not change with concentration (Figure 9C). This amount of dimer is consistent with that formed by Cu(*o*-phen)$_2$ treatment of NEM-actin (Figure 1B). Thus, Cu(*o*-phen)$_2$ is not forming a stable complex with two actins, and its oxidative cross-linking of actin to form an intermolecular dimer is due to a transient, collisional actin−actin complex.

In control experiments, we conducted sedimentation velocity of purified cross-linked antiparallel actin dimer (Figure 9D). The dimer ran as a single monodisperse species with a molecular mass of 85 kDa, twice that of the actin monomer, and with a sedimentation coefficient of 4.6 S, much higher than that of the species identified as the monomer. Thus, the species identified as a monomer in Figure 8A−C is not a dimer but is indeed a monomer.

We have shown that although G-actin can be cross-linked to an antiparallel dimer in high yields, no detectable dimer exists in solution without the presence of a cross-linking agent. That is, dimer formation is energetically unfavorable such that cross-linking occurs only during collisional encounters between two actin monomers. Therefore, the general assumption that the ability to form cross-linked actin dimers implies the presence of stable actin dimers in solution[2−4,7,9,35] is not valid.

F-Actin Polymeric Filaments. Mature actin filaments, i.e., those formed after polymerization for more than 30 min, were treated with Cu(*o*-phen)$_2$ as a function of time, and the amount

of cross-linked antiparallel dimer formation was recorded in Figure 4B. The amount of dimer formed was much smaller and the rate of formation much slower than those for monomeric actin (Figure 4A). For monomeric actin, the maximal yield was between 40 and 54% (Figures 3−5), and this was achieved in ∼10 min (Figure 4A). For filamentous actin, the maximal yield was not reached even after 60 min, at which point the yield was only 15%. This behavior is consistent with the conclusion described above that the cross-linked dimer forms during the oxidation of collisional complexes of two actin monomers and that for F-actin the monomers cross-linked are those in equilibrium, via continuous treadmilling, with filamentous actin. As actin monomers treadmill off of actin filaments, they collide and are cross-linked by Cu(*o*-phen)$_2$. This is a slow and continuous process that reveals itself as a slow and continuous accumulation of cross-linked dimer (Figure 4B).

Previous work has shown close to zero cross-linked antiparallel actin formation for mature actin filaments using cross-linking reagents.[2,7,9,10] This is not surprising because the cross-linking reaction was conducted for only 0.5−2 min.

DISCUSSION

The antiparallel actin dimer model of in vitro actin polymerization is based on the observation that in the early stages of polymerization a relatively large amount of cross-linked antiparallel actin dimers is formed by divalent chemical cross-linking agents.[2−4,7,9,10] Furthermore, as the polymerization proceeds and leads to mature actin filaments, it is observed that the level of formation of such dimers is diminishingly small.[2−4,7,9,10] These studies thereby propose the existence of stable antiparallel actin dimers that are incorporated into the growing actin filament and then reorient to normal parallel dimers within the filament as the filament matures.

Our results demonstrate that although we can obtain high yields of disulfide cross-linked antiparallel actin dimer there is no stable actin dimer present. This leads us to propose that this antiparallel species is cross-linked only during transient collisional encounters. In this scheme, at the early stages of polymerization, where there are relatively large amounts of unpolymerized monomers, the probability of collisional encounters is high, and this results in a relatively high yield of cross-linked dimer in the presence of cross-linker. As polymerization proceeds, the monomer concentration decreases and there are fewer intermonomer collisions, and thus, fewer cross-linked antiparallel dimers formed. Therefore, cross-linked antiparallel dimer formation is only indirectly related to the polymerization process. Consequently, our conclusions question the validity of the antiparallel actin dimer model of actin polymerization, although we cannot entirely rule it out. However, there are alternate models proposed in the literature that do not invoke the presence of actin antiparallel dimers.[5,6,8,11]

There are other studies that have been used to support the antiparallel actin dimer model of actin polymerization. During the polymerization of actin labeled at its "tail" with a pyrene probe, there is transient formation of excimer fluorescence indicative of a stable tail-to-tail actin dimer.[7,10,35] However, such a species could be formed only in the presence of polylysine,[7,10,35] a polycation known to induce acidic protein, including actin, dimerization, and oligomerization.[36−39] Furthermore, hydrophobic interaction between the pyrene moieties might also contribute to dimer formation, which has been found to be the case for similarly labeled yeast actin.[10]

Another study has developed a unique antibody specifically reactive to the actin antiparallel dimer to identify such a species in cells.[40] Cells were fixed, i.e., cross-linked, with formaldehyde before the antibody was applied. However, during the period of cross-linking, any collisionally formed antiparallel actin dimers would be trapped and positively react with the antibody. Thus, reaction with the antibody does not unequivocally establish the existence of a stable antiparallel actin species free in solution. These authors also demonstrated that in vitro bundles of actin filaments reacted with the antibody, demonstrating interfilament antiparallel actin−actin interactions.[40] The results from our work do not have any bearing on this conclusion. It is not determined from the work on cells whether the identified antiparallel dimers are free in solution or are intra- or inter-actin filament.[40]

AUTHOR INFORMATION

Corresponding Author

*Boston Biomedical Research Institute, 64 Grove St., Watertown, MA 02472. Telephone: (617) 658-7813. Fax: (617) 972-1953. E-mail: graceffa@bbri.org.

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Notes

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ABBREVIATIONS

ASSA, Cys374−Cys374 disulfide cross-linked antiparallel actin dimer; Cu(*o*-phen)$_2$, Cu^{2+}-(*o*-phenantroline)$_2$ SO_4^{2-}; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; AUC, analytical ultracentrifugation; Mops, 3-(*N*-morpholino)propanesulfonic acid; CP-A, carboxypeptidase A; TMR-actin, actin modified at Cys374 with tetramethylrhodamine-5-maleimide; au, arbitrary units.

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