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DISULFIDE CROSS-LINKED ANTI-PARALLEL ACTIN DIMER[#]

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Abstract

Oxidation of actin monomer (G-actin) with copper α -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. anti-parallel, actin dimer. Analytical ultracentrifugation profiles of G-actin can be accounted for by 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, 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 actin monomer concentration is high) cross-linked anti-parallel actin dimers are formed in relatively high yield whereas none are formed at later stages of polymerization. The present findings raise questions concerning the validity of the anti-parallel 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, to 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 referred to as 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*, actin polymerization 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 light scattering or 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, more straight, 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).

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¹Abbreviations used: ASSA, Cys374-Cys374 disulfide cross-linked anti-parallel actin dimer; Cu(α -phen)₂, Cu²⁺(α -phenanthroline)₂ SO₄²⁻; 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; a.u., arbitrary units.

There are several models which have been put forth to explain this transition between actin filament types. One model is that, early in polymerization, the filament incorporates anti-parallel actin dimers, which disrupt the smooth addition of subsequent actin monomers (2-4, 7, 9, 10). This model further stipulates that, with time, these anti-parallel 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 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 anti-parallel actin dimers (5, 6, 8, 11).

The basis of the anti-parallel actin dimer model is the observation that in the early stages of polymerization one is able to form cross-linked anti-parallel actin dimers by cross-linking actin Cys374 of one molecule to actin Cys374 of another via di-valent chemical cross-linkers (2-4, 7, 9, 10). At later stages of polymerization, during the formation of mature filaments, cross-linked anti-parallel 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. The present 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 assumption is not true, which adds caution to the validity of the anti-parallel actin dimer model of actin polymerization.

MATERIALS AND METHODS

Actin Preparation

Rabbit skeletal muscle actin was prepared as described (12). Some actin was modified with NEM (13) or digested with *Staphylococcus aureus* V8 protease (14) as reported. Actin was labeled at Cys374 with tetramethylrhodamine-5-maleimide (Invitrogen) as described (15, 16). Actin was generally stored for up to about 2 weeks on ice as the monomer in low ionic strength G-buffer (2 mM Mops, 0.2 mM CaCl₂, 0.2 mM ATP, 0.01% NaN₃, pH 7.5). Actin concentration was determined from the optical density at 290 nm minus that at 320 nm using an extinction coefficient $A_{290\text{nm}}^{1\%} = 6.3 \text{ cm}^{-1}$ (17). Actin was polymerized by adding to actin (in G-buffer) NaCl to 40 mM and MgCl₂ to 2 mM (F-buffer).

Sulfhydryl Content

Total sulfhydryl content of actin denatured in 4 M guanidine hydrochloride was determined colorimetrically by reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (Sigma) (18).

Oxidation by Copper Phenanthroline

A stock solution of 5 mM Cu(α -phen)₂ was prepared by dissolving α -phenanthroline (Fisher Scientific) to 10 mM in 5 mM CuSO₄. The reaction between Cu(α -phen)₂ and actin was initiated by adding 1 mM Cu(α -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 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 – 0.04 and reacted for 0.5 – 3 hours at room temperature. At the end of the

reaction time $\text{Cu}(o\text{-phen})_2$ was added at a $\text{Cu}(o\text{-phen})_2$ /actin molar ratio of 1.0 and reacted for 0.5 hour at room temperature. This reaction was terminated with 5 mM NEM whereupon SDS-PAGE was run.

Gel Filtration Chromatography

Gel filtration chromatography of actin or $\text{Cu}(o\text{-phen})_2$ -treated actin was carried out on a 16 mm $\times$ 60 cm Pharmacia Superdex 200 column. Before application to the column the treated actin was dialyzed against G-buffer to remove the $\text{Cu}(o\text{-phen})_2$. 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, 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 on-line 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 analyzed essentially as detailed previously (21). The program SEDANAL (22) was used to calculate the sedimentation velocity weight average sedimentation coefficients, the program SEDNTERP (23) was used to estimate hydration and convert sedimentation coefficients to standard condition of 20 °C in water, $s_{20,w}$, and the program SEDANAL (22) was used to test sedimentation velocity data for interacting models.

Actin Polymerization

Polymerization of actin or ASSA (both in G-buffer $\pm$ 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/MgCl₂ solution such that the final concentration was 40 mM NaCl, 2 mM MgCl₂. The final concentration of actin (monomer-equivalent) was 2 μM . Samples were incubated for 1 h at 20 °C before initiation of polymerization in order to insure 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 that of the actin monomer at 42 kDa. Treatment of the Cu(*o*-phen)₂-oxidized actin with DTT, a reagent which 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.

It can be concluded that the actin dimer bond is a disulfide bond between Cys374 of one molecule and Cys374 of another, i.e. a tail-to-tail or an anti-parallel dimer, for the following four reasons. (1) Cu(*o*-phen)₂ treatment of actin modified with NEM, which specifically blocks Cys374 (30, 31), showed a greatly diminished actin dimer band on SDS-PAGE (Figure 1B). (2) Carboxypeptidase A (CP-A) treated actin, which has Phe375, Cys374, and Lys373 removed (19), also resulted in a reduction of Cu(*o*-phen)₂-generated dimer formation (Figure 2). The reduction increased 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 anti-parallel 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 which contains a cystine residue containing two Cys374 residues, as determined by Edman degradation sequencing (not shown). A similar disulfide cross-linked anti-parallel actin dimer has been observed after freeze-thawing of actin (32) or of disulfide-modified actin (33).

The maximum 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 maximum 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 due to Cu²⁺-induced aggregation. The maximum yield was reached in approximately 10 minutes (Figure 4A). The dimer yield was relatively insensitive to pH values from 7.0 to 8.0 and to temperatures between 0 °C to 25 °C. The reaction yield did increase with actin concentration, reaching 50% dimer at about 100 μM actin (Figure 5).

It was not convenient to study the ionic strength-dependence of the actin dimerization reaction in the monomeric state since the addition of salts polymerize actin. Therefore we examined the reaction at low ionic strength, 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 strength which is known to reversibly depolymerize F-actin (34). From this previous work (34) we speculated that NH₄SCN might efficiently depolymerize actin. Indeed, at a NH₄SCN 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 (below, Figure 9B). Reaction of actin with Cu(*o*-phen)₂ as a function of NH₄SCN concentration resulted in a dimer yield ranging from 50% at 0 M NH₄SCN to 51% at 0.1 M NH₄SCN to 64% at 0.3 M NH₄SCN to 68% at 0.4 M NH₄SCN. Thus cross-linked dimer formation increases moderately with increasing ionic strength.

It is curious why the cross-linked actin dimer formation is limited to around 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. In order to test this we separated Cu(*o*-phen)₂-oxidized (in G-buffer) actin into 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 NH₄SCN (Figure 6). This suggests that 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 above hypothesis 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 heterogeneous mixture of two actin molecules but that Cu(*o*-phen)₂ was reacting with actin Cys374 to form either an intermolecular disulfide bond or oxidizing Cys374 to a DTT-irreversible oxidation state. Indeed the sulfhydryl content of 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 I). That is, the monomer lost two sulfhydryls out of a total of five for actin, which were not recovered by DTT treatment. The dimer lost only one sulfhydryl/monomer component, which was recovered by DTT treatment. Cu(*o*-phen)₂ -oxidation of actin sulfhydryls to a DTT-insensitive oxidation state was less when Cu(*o*-phen)₂ treatment was carried out in the presence of NH₄SCN 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 NH₄SCN.

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 NH₄SCN. The polymerization profile of actin purified in the presence or absence of NH₄SCN was identical (not shown), indicating no irreversible deleterious effect of NH₄SCN on actin polymerization. These results demonstrate that reduced ASSA is fully polymerization competent, which is consistent with the above results (Table I) that show that for ASSA only the cysteine involved in the disulfide bond is modified by the Cu(*o*-phen)₂ treatment.

Analytical Ultracentrifugation—The question arises as to whether or not actin, under the conditions that result in a high yield of cross-linked dimer, forms a stable dimeric species and how much. Cross-linking cannot reveal this information since it shifts the equilibrium between 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 which 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 where high yields of cross-linked dimers were formed (Figure 9A). Curve fitting was carried out using a non-ideal, single component model. Attempts to fit to a non-ideal monomer-dimer model returned a value of 0.0 for the dimerization constant. Very similar results were obtained for actin in 0.3 M NH₄SCN (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 in going from 0 to 0.3 M NH₄SCN. In 0.3 M NH₄SCN there was a small amount of material (< 1% of the actin) sedimenting at an

apparent sedimentation coefficient > 10 which most likely corresponds to actin oligomers or aggregates.

We wished to test the possibility that $\text{Cu}(\text{o-phen})_2$ was inducing the formation of a stable complex in which the two phenanthroline moieties of $\text{Cu}(\text{o-phen})_2$ bridged two actin monomers whereupon the monomers would be oxidized to an intermolecular disulfide dimer. Therefore we measured sedimentation velocity of NEM-modified actin, which does not become $\text{Cu}(\text{o-phen})_2$ -oxidized to a dimer (Figure 1B), in the presence of equimolar $\text{Cu}(\text{o-phen})_2$. 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 which did not change with concentration (Figure 9C). This amount of dimer is consistent with that formed by $\text{Cu}(\text{o-phen})_2$ treatment of NEM-actin (Figure 1B). Thus $\text{Cu}(\text{o-phen})_2$ 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 ran sedimentation velocity of purified cross-linked anti-parallel actin dimer (Figure 9D). The dimer ran as a single monodisperse species with a molecular weight 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 - 8C is not a dimer but is indeed a monomer.

We have shown that although G-actin can be cross-linked to an anti-parallel 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 $\text{Cu}(\text{o-phen})_2$ as a function of time and the amount of cross-linked anti-parallel dimer formation recorded in Figure 4B. The amount of dimer formed was much less and the rate of formation much slower than that for monomeric actin (Figure 4A). For monomeric actin the maximum yield was between 40 - 54 % (Figures 3 - 5) and this was achieved in about 10 min (Figure 4A). For filamentous actin the maximum yield was not reached even after 60 min where the yield was only 15 %. This behavior is consistent with our above conclusion 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 $\text{Cu}(\text{o-phen})_2$. 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 anti-parallel actin formation for mature actin filaments using cross-linking reagents (2, 7, 9, 10). This is not surprising since the cross-linking reaction was carried out for only 0.5-2 min.

DISCUSSION

The anti-parallel 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 anti-parallel actin dimers are formed by di-valent 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 formation of such dimers is diminishingly small (2-4, 7, 9, 10). These studies thereby propose the existence of stable anti-parallel 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 anti-parallel actin dimer there is no stable actin dimer present. This leads us to propose that this anti-parallel 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 less inter-monomer collisions and thus less cross-linked anti-parallel dimers formed. Therefore cross-linked anti-parallel dimer formation is only indirectly related to the polymerization process. Consequently, our conclusions question the validity of the anti-parallel 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 anti-parallel dimers (5, 6, 8, 11).

There are other studies that have been used to support the anti-parallel actin dimer model of actin polymerization. During the polymerization of actin labeled at its “tail” with a pyrene probe, there is a transient formation of excimer fluorescence indicative of a stable tail-to-tail actin dimer (7, 10, 35). However such a species could only be formed 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 anti-parallel dimer in order to identify such a species in cells (40). Cells were fixed, i.e. cross-linked, with formaldehyde before applying antibody. However during the period of cross-linking, any collisionally-formed anti-parallel 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 anti-parallel actin species free in solution. These authors also demonstrated that *in vitro* bundles of actin filaments reacted with the antibody demonstrating inter-filament anti-parallel 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 anti-parallel dimers are free in solution or are intra- or inter-actin filament (40).

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Figure 1. SDS-PAGE of Cu(*o*-phen)₂-oxidized G-actin. A, molecular weight standards and 100 μ M actin treated with equimolar Cu(*o*-phen)₂ for 1 hr at room temperature in G-buffer; B, 83 μ M NEM-actin treated with equimolar Cu(*o*-phen)₂ for 1 hr at room temperature in G-buffer. D = dimer, M = monomer.

Figure 2.

SDS-PAGE of $\text{Cu}(\text{o-phen})_2$ -oxidized, carboxypeptidase A-treated G-actin. CP-A-treated actin at $153 \mu\text{M}$ in G-buffer reacted with equimolar $\text{Cu}(\text{o-phen})_2$ for 30 min at room temperature in G-buffer. Lane A, control actin; lanes B, C, & D, actin treated with CP-A for 1 hr at room temperature at actin/CP-A molar ratio of 100, 50, & 25, respectively; lanes E, F, G, & H, actin treated with CP-A at actin/CP-A molar ratio of 25 for 0.5, 1, 2, & 3 hr, respectively. D = dimer, M = monomer.

Figure 3.

G-actin dimer formation as a function of $\text{Cu}(\text{o-phen})_2/\text{actin}$ molar ratio. Percentage actin as dimer after reaction of $25\ \mu\text{M}$ actin with $\text{Cu}(\text{o-phen})_2$ at room temperature for 10 min in G-buffer. Dimer quantified from densitometry of SDS-PAGE.

Figure 4.

Time dependence of actin dimer formation upon $\text{Cu}(\text{o-phen})_2$ oxidation. Percentage actin as dimer after reaction of $25\ \mu\text{M}$ actin with equimolar $\text{Cu}(\text{o-phen})_2$ at room temperature in G-buffer (A) or F-buffer (B). In (B) actin was polymerized in F-buffer for 45 min at room temperature before adding $\text{Cu}(\text{o-phen})_2$. Dimer quantified from densitometry of SDS-PAGE.

Figure 5.

G-actin concentration dependence of actin dimer formation upon oxidation with $\text{Cu}(\text{o-phen})_2$. Percentage actin as dimer after reaction of actin with equimolar $\text{Cu}(\text{o-phen})_2$ for 10 min at room temperature in G-buffer. Dimer quantified from densitometry of SDS-PAGE.

Figure 6. Gel chromatography of $\text{Cu}(\text{o-phen})_2$ -oxidized G-actin. Superdex 200 gel chromatography in G-buffer + 0.3 M NH_4SCN . D = dimer, M = monomer. Inset = SDS-PAGE of column fraction aliquots.

Figure 7.

Re-oxidation by $\text{Cu}(o\text{-phen})_2$ of purified and reduced actin monomer and dimer. $\text{Cu}(o\text{-phen})_2$ -oxidized (for 1 hr) G-actin separated into monomer and dimer was followed by DTT reduction and re-oxidation by $\text{Cu}(o\text{-phen})_2$. A, monomer; B, dimer; C, actin control. Re-oxidation was of $67\ \mu\text{M}$ G-actin with equimolar $\text{Cu}(o\text{-phen})_2$ for 15 min on ice in G-buffer. M = monomer band, D = dimer band.

Figure 8. Polymerization of actin, ASSA and reduced ASSA at 2 μ M actin (monomer-equivalent) concentration. Polymerization vs time at 20 $^{\circ}$ C in 40 mM NaCl, 2 mM MgCl_2 , $\pm$ 12.5 mM DTT in G-buffer, monitored by the 575nm fluorescence of 5% of the TMR-actin reporter molecule. Actin +DTT (red), ASSA (green), ASSA +DTT (black).

Figure 9.

Analytical ultracentrifugation sedimentation velocity of actin. (A) actin in G-buffer + 5 mM DTT, (B) actin in G-buffer + 0.3 M NH_4SCN + 5 mM DTT, (C) NEM-actin in G-buffer + $\text{Cu}(o\text{-phen})_2$ equimolar to NEM-actin, and (D) purified cross-linked anti-parallel actin dimer in G-buffer. The concentration-normalized sedimentation distribution function (y-axis) is plotted *versus* the apparent sedimentation coefficient (x-axis). All runs conducted at 20 °C.

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

^aOxidation reaction in G-buffer (column A) or G-buffer with 0.3 M NH₄SCN (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.