

# Probing Actin Polymerization by Intermolecular Cross-linking

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**Abstract.** We have used *N,N*-1,4-phenylenebismaleimide, a bifunctional sulfhydryl cross-linking reagent, to probe the oligomeric state of actin during the early stages of its polymerization into filaments. We document that one of the first steps in the polymerization of globular monomeric actin (G-actin) under a wide variety of ionic conditions is the dimerization of a significant fraction of the G-actin monomer pool. As polymerization proceeds, the yield of this initial dimer ("lower" dimer with an apparent molecular mass of 86 kD by SDS-PAGE [LD]) is attenuated, while an actin filament dimer ("upper" dimer with an apparent molecular mass of 115 kD by SDS-PAGE [UD] as characterized [Elzinga, M., and J. J. Phelan. 1984. *Proc. Natl. Acad. Sci. USA.* 81:6599-6602]) is formed. This shift from LD to UD occurs concomitant with formation of filaments as assayed by *N*-(1-pyrenyl)iodoacetamide fluorescence enhancement and electron microscopy. Isolated cross-linked LD does not form filaments, while isolated cross-linked UD will assemble into filaments indistinguishable from those

polymerized from unmodified G-actin under typical filament-forming conditions. The presence of cross-linked LD does not effect the kinetics of polymerization of actin monomer, whereas cross-linked UD shortens the "lag phase" of the polymerization reaction in a concentration-dependent fashion. Several converging lines of evidence suggest that, although accounting for a significant oligomeric species formed during early polymerization, the LD is incompatible with the helical symmetry defining the mature actin filament; however, it could represent the interfilament dimer found in paracrystalline arrays or filament bundles. Furthermore, the LD is compatible with the unit cell structure and symmetry common to various types of crystalline actin arrays (Aebi, U., W. E. Fowler, G. Isenberg, T. D. Pollard, and P. R. Smith. 1981. *J. Cell Biol.* 91:340-351) and might represent the major structural state in which a mutant  $\beta$ -actin (Leavitt, J., G. Bushar, T. Kakunaga, H. Hamada, T. Hirakawa, D. Goldman, and C. Merril. 1982. *Cell.* 28:259-268) is arrested under polymerizing conditions.

**O**VER the past two decades much has been learned about the mechanism of actin polymerization *in vitro* (for recent reviews see Korn, 1982; Frieden, 1985). The fundamental work of Oosawa and co-workers (see Oosawa and Asakura, 1975) has explained many of the experimental findings in terms of a nucleation-condensation mechanism. During nucleation, monomers associate to form unstable structures that define only a single helical contact; however, once an oligomer forms that defines both contacts of the actin helix, the stability of the resulting structure is greatly increased and rapid elongation from these "nuclei" ensues. Such a nucleation-condensation process is supported by the observation that the rapid polymerization into filaments is always preceded by a concentration-dependent "lag phase" (see Oosawa and Asakura, 1975; Wegner and Engel, 1975; Tobacman and Korn, 1983; Cooper et al., 1983a; Frieden and Goddette, 1983; Frieden, 1983; see also Matsudaira et al., 1987), which may reflect the time needed for nuclei to form. Alternatively, the occurrence of a lag phase could also be explained by a simple activation step or even by some combination of activation and nucleation.

Several groups have demonstrated independently, using a variety of techniques, that activation of the monomer from a globular monomeric actin (G-actin)<sup>1</sup> conformation to a filamentous polymeric actin (F-actin)-like conformation precedes filament formation (Cooper et al., 1983a; Pardee and Spudich, 1982; Rich and Estes, 1976; Rouayrenc and Travers, 1981) when actin is polymerized in the presence of MgCl<sub>2</sub> or KCl. In addition, Newman et al. (1985) and Mozo-Villarias and Ware (1985) have shown that the diffusion coefficient of actin is decreased upon Mg<sup>2+</sup> binding to actin below its critical concentration (i.e., the minimum actin concentration required for filament formation), suggesting that Mg<sup>2+</sup> might induce an initial oligomerization of actin. Under similar conditions Goddette et al. (1986) have reported that actin will form "nonproductive" dimers that do not par-

1. *Abbreviations used in this paper:*  $\beta^*$ -actin, single-site mutant  $\beta$ -actin; F-actin, filamentous polymeric actin; G-actin, globular monomeric actin; LD, lower dimer with an 86-kD apparent molecular mass by SDS-PAGE; 1,2-PBM, *N,N*-1,2-phenylenebismaleimide; 1,4-PBM, *N,N'*-1,4-phenylenebismaleimide; pyrene, *N*-(1-pyrenyl)iodoacetamide; UD, upper dimer with a 115-kD apparent molecular mass by SDS-PAGE.

participate in the assembly process. Utilizing x-ray scattering from a synchrotron source, Matsudaira et al. (1987) were able to probe the degree of oligomerization during the early stages of polymerization with high temporal (i.e., 10–100 ms) resolution. According to their measurements, most of the actin pool dimerizes during the first second of polymerization; it is these dimers which represent the major building blocks for subsequent rapid filament formation.

It was found some time ago that the cross-linking reagent *N,N'*-1,4-phenylenebismaleimide (1,4-PBM) specifically reacts with actin filaments yielding a variety of cross-linked oligomers (see Knight and Offer, 1978). Subsequently, a cross-linked dimer with an apparent molecular mass of 115 kD by SDS-PAGE (UD), obtained from filaments reacted with 1,4-PBM, has been isolated and characterized (Grumet and Lin, 1980; Mockrin and Korn, 1981, 1983) and found to resemble F-actin in its structural and functional properties. More recently, this cross-link has been mapped: it covalently links Cys<sub>374</sub> on one subunit to Lys<sub>191</sub> on the other subunit (Elzinga and Phelan, 1984). Evidence has been given (Knight and Offer, 1978) that this dimer is formed along the “long-pitch” and not along the “genetic” helix (for actin helix nomenclature see Egelman [1985] or Aebi et al. [1986]). This, in turn, may explain the finding by Gilbert and Frieden (1983) that this dimer is a suboptimal nucleus in the sense that, while reduced, there is still a lag phase observed when a monomer solution under polymerizing conditions is seeded with this cross-linked dimer. This would be expected, as a long-pitch helix dimer only defines the intersubunit contacts along the two long-pitch helices but not between them.

Here we report the occurrence of an additional 1,4-PBM cross-linked actin dimer with an apparent molecular mass of 86 kD by SDS-PAGE (LD) that is observed immediately upon the addition of polymerizing salts to G-actin, is present in its highest yield during the lag phase, and is consumed as filaments form. We have isolated and characterized this dimer and found that its properties more closely resemble those of G-actin than those of F-actin. We further suggest, on the basis of several converging lines of evidence, that this dimer, while possibly a filament precursor, does not represent a filament dimer (i.e., it is in a structural configuration not found in a mature filament). The LD might, in fact, define an interfilament contact (i.e., a dimer shared between adjacent filaments) such as in paracrystal-like arrays or bundles of F-actin, and the major contact, across the dyad axis, in crystalline actin sheets induced by stoichiometric amounts of gadolinium (dos Remedios and Dickens, 1978; Aebi et al., 1981).

## Materials and Methods

### Materials

All chemicals were of reagent grade. ATP (sodium salt, grade I) and dimethylformamide (DMF) were purchased from Sigma Chemical Co. (St. Louis, MO). 1,4-PBM and *N,N'*-1,2-phenylenebismaleimide (1,2-PBM) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further recrystallization (see Knight and Offer, 1978). *N*-(1-pyrenyl)-iodoacetamide (pyrene) was obtained from Molecular Probes (Junction City, OR).

### Actin

Rabbit skeletal muscle actin was isolated from an acetone powder by the

method of Spudich and Watt (1971) and further purified by gel filtration on a 2.5 × 100 cm Sephadex G-200 column (Pharmacia Fine Chemicals, Piscataway, NJ) as described by MacLean-Fletcher and Pollard (1980). The resulting G-actin peak fractions (typically 1.0–1.5 mg/ml) were stored in buffer A (2.5 mM imidazole, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP, 0.1 mM dithiothreitol [DTT], 0.005% NaN<sub>3</sub>, pH 7.5) at 4°C.

### Cross-linking during Actin Polymerization

A 5-mM stock solution of 1,4- or 1,2-PBM was prepared by dissolving the solid in 100% DMF. This stock solution was kept on ice during the experiment. Polymerization was initiated by the addition of the appropriate type and amount of salt(s) (see Results) to G-actin at a concentration of 1 mg/ml that had been extensively dialyzed against buffer B (2.5 mM imidazole, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP, 0.005% NaN<sub>3</sub>, pH 7.5). Immediately before (i.e., <30 s) each time point assayed by cross-linking, an aliquot of the 1,4- or 1,2-PBM stock solution was diluted to twice the final cross-linker concentration with buffer C (10 mM Na-borate containing the type and amount of salt(s) used for polymerization, pH 9.2). At each time point a 20- $\mu$ l aliquot of the PBM-containing buffer C solution was added to a 20- $\mu$ l aliquot of polymerizing actin. 1 min later the cross-linking reaction was quenched by the addition of 20  $\mu$ l of 3× SDS-PAGE sample buffer containing 5%  $\beta$ -mercaptoethanol. The presence of cross-linked actin oligomers was assayed by SDS-PAGE as described by Laemmli (1970). The final molar concentration of 1,4- or 1,2-PBM during the cross-linking reaction was half the actin molar concentration (i.e., a cross-linker/actin molar ratio of 0.5:1), unless otherwise stated. The concentration of DMF during cross-linking was always <0.5%. The “operational zero” time point in each experiment was determined by the time (typically 3–10 s) it took to cross-link the first aliquot after the addition of salt.

### Preparation and Isolation of Cross-linked LD and UD

To prepare and isolate LD, 5–10 mg of actin (i.e., 3–5 ml of G-actin at a concentration of 1–1.5 mg/ml) were cross-linked with 1,4-PBM at a cross-linker/protein ratio of 0.5:1 under conditions that yielded LD exclusively (i.e., no UD) as the cross-linked species. Typically this was achieved by cross-linking immediately after salts were added to G-actin at a final concentration of 2 mM MgCl<sub>2</sub>/50 mM KCl or 2 mM CaCl<sub>2</sub>. The cross-linking reaction was quenched after 5 min by the addition of a 100-fold molar excess of  $\beta$ -mercaptoethanol relative to 1,4-PBM. The cross-linked material was then dialyzed against buffer A (see above) for 48 h with several buffer changes. Finally, the sample was centrifuged at 185,000 g for 2 h, and the supernatant was loaded onto a Sephadex G-200 column (2.5 × 100 cm) equilibrated in buffer A. The peak elution between the void and the monomer position was pooled and assayed for purity by SDS-PAGE.

The preparation and isolation of UD was done analogously, except that the actin was cross-linked under conditions that yielded UD exclusively. Typically this was achieved by cross-linking actin 10 min after the onset of polymerization with 2 mM MgCl<sub>2</sub>/50 mM KCl.

### Pyrene Fluorescence Measurements

Monitoring actin polymerization by the change in the fluorescence signal of pyrene actin was performed as described previously (see Cooper et al., 1983b). Pyrenated actin accounted for 9% of the total actin in Fig. 1 and 5% of the total actin in Fig. 8.

### Molecular Mass Determination of LD and UD

To determine the true molecular masses ( $M_r$ ) of LD and UD, sedimentation equilibrium centrifugation was performed in an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Fullerton, CA) equipped with absorption optics and a photoelectric scanner. Isolated cross-linked LD or UD samples were equilibrated in 2.5 mM imidazole, 0.2 mM CaCl<sub>2</sub>, 0.005% NaN<sub>3</sub>, pH 7.5, at 4°C at protein concentrations ranging from 0.1 to 0.25 mg/ml, and run in 12-mm or 30-mm double-sector cells filled to a column height of 2–3 mm at speeds varied between 12,000 and 14,000 rpm. The baseline absorbance was determined by analysis of the data using a linear regression program (written by Dr. H. Berger at the Biozentrum in Basel, Switzerland), which adjusted the baseline so as to obtain the best linear fit of  $\ln A$  vs.  $r^2$ . A partial specific volume of 0.73 cm<sup>3</sup>/g was used for all calculations.

## Preparation of Gd<sup>3+</sup>-induced Crystalline Actin Sheets

These were prepared as described previously for *Acanthamoeba* actin (Aebi et al., 1981), except that for rabbit skeletal muscle actin the amount of KCl in the crystallization buffer had to be reduced from 100–150 mM to 10–25 mM, and the optimal molar ratio of Gd<sup>3+</sup>/actin was found to be 5:1.

## Electron Microscopy

For negative staining, 3- $\mu$ l aliquots were adsorbed for 30 s to carbon-coated 400 mesh/inch copper grids that were rendered hydrophilic by glow discharge for 10 s in a reduced atmosphere of air (see Aebi and Pollard, 1987). After blotting off excess sample the grids were washed for 1–5 s on one drop of distilled water and stained for a total of 15 s on several drops of 0.75% uranyl formate, pH 4.25 (Aebi et al., 1981; Smith et al., 1983). Glycerol spraying/rotary metal shadowing of monomer, purified cross-linked LD and UD was performed as described previously (Fowler and Aebi, 1983) by adding glycerol to 30% final to the samples (typically at 0.1 mg/ml) equilibrated in buffer A (see above). Samples were examined in a Zeiss EM 10C electron microscope operated at an accelerating voltage of 80 kV. Micrographs were recorded at 50,000 $\times$  nominal magnification on Kodak 4489 or SO-163 electron microscope film and developed for 4 min in Kodak D-19 diluted 1:2 with water. The magnification was calibrated as described by Wrigley (1968) using negatively stained catalase crystals as the calibration standard.

## Results

### A Cross-linking Assay for Actin Polymerization

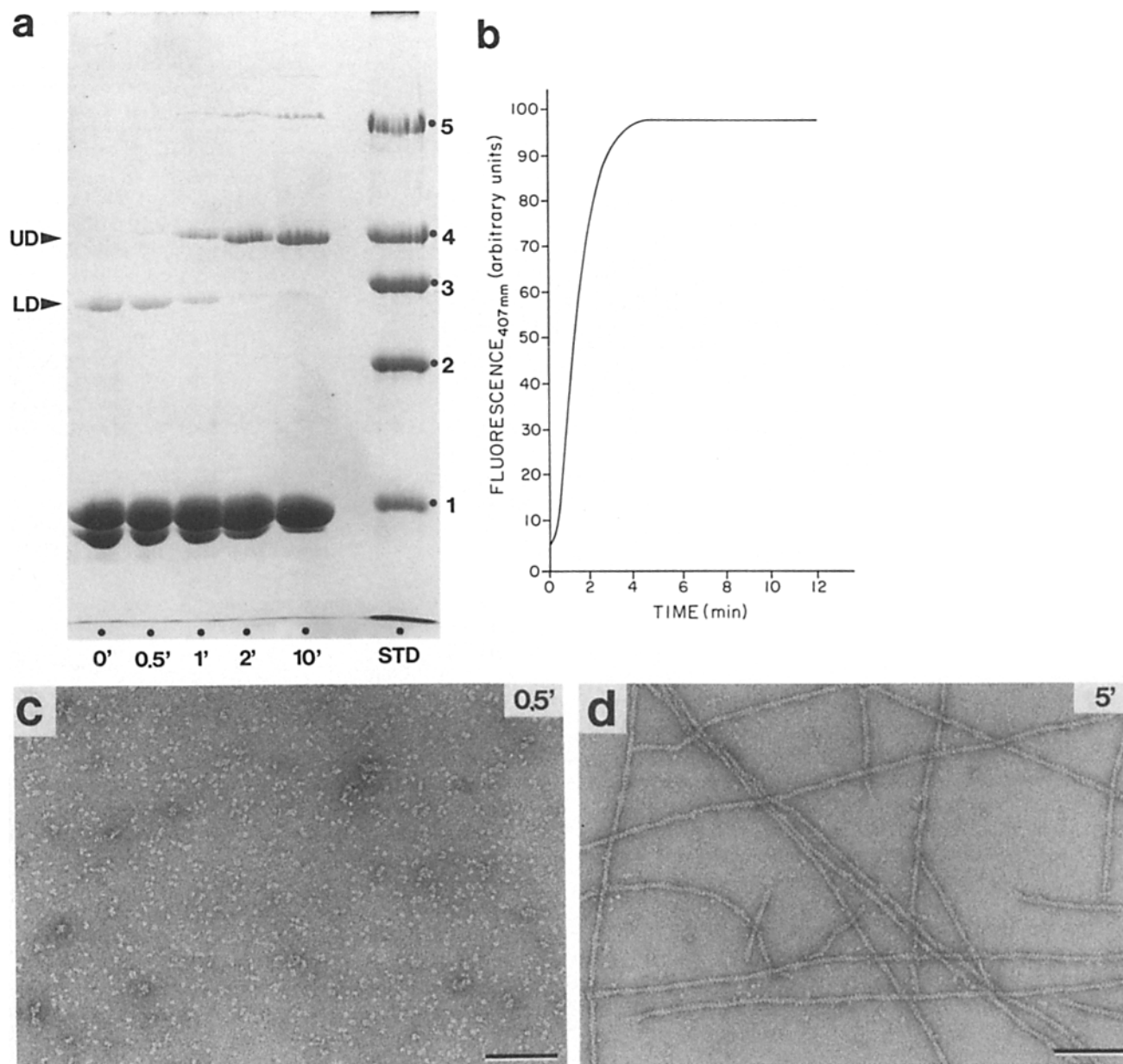
In the course of trying to generate different intermolecular cross-links within the actin filament to be used to constrain the orientation of the actin subunit within three-dimensional models of the actin filament (see Smith et al., 1983; Egelman, 1985; Aebi et al., 1986), we have used the sulfhydryl cross-linking reagent 1,4-PBM, which has been shown to specifically cross-link adjacent subunits in actin filaments (Knight and Offer, 1978; Elzinga and Phelan, 1984). As illustrated in Fig. 1 *a*, when G-actin is polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl, and aliquots are removed at different times and cross-linked with 1,4-PBM as described in Materials and Methods, the only cross-linked oligomeric species observed at early time points (i.e.,  $\leq 30$  s at 1 mg/ml actin) migrates on SDS-PAGE with an apparent molecular mass of 88 kD. This band is observed as rapidly as a sample can be cross-linked after the onset of polymerization (see Materials and Methods). With increasing time this 88-kD band gradually disappears concomitant with the appearance of a second species with an apparent molecular mass of 115 kD (Fig. 1 *a*). This behavior suggests that the 88-kD species initially formed is consumed during the course of polymerization. It should be stressed, however, that this change in cross-linking preference by no means proves a direct conversion of the 88-kD species to the 115-kD species; that is, the breakdown of the 88-kD species to monomer and the subsequent formation of the 115-kD species from monomer cannot be discounted. Both of these bands were observed previously when actin filaments were cross-linked with 1,4-PBM (Mockrin and Korn, 1981). However, in that report, though the upper band was interpreted as a filament dimer (Knight and Offer, 1978), the lower band was assumed to be either a differently cross-linked species of the same filament dimer, or an intramolecularly cross-linked monomer. In addition, a splitting of the monomer band into two electrophoretic species was observed upon cross-linking (Fig. 1 *a*). The most likely explanation for this behavior is that some monomer modification, possibly intramolecular cross-linking, has occurred, resulting in

an aberrant mobility on SDS-PAGE. Since we shall show below that both the 88-kD as well as the 115-kD species (see Mockrin and Korn, 1983) are actin dimers, we shall refer to the species corresponding to the lower gel band (apparent molecular mass, 88 kD) as the LD and the species corresponding to the upper gel band (apparent molecular mass, 115 kD) as the UD.

We have compared the shift of LD to UD as monitored by our cross-linking assay with other polymerization assays, and, as illustrated in Fig. 1 *b* versus *a*, the time of the shift from LD to UD correlates remarkably well with the transition from the lag to the elongation phase as assayed by the fluorescence enhancement of pyrene actin (Cooper et al., 1983*b*) under identical conditions. Additionally, we have assayed the state of polymerization of actin at various times by electron microscopy. When the LD is the predominant cross-linked species (i.e., at 30 s after the onset of polymerization of 1 mg/ml of G-actin with 2 mM MgCl<sub>2</sub>/50 mM KCl), only small oligomers are found in the electron microscope (Fig. 1 *c*), whereas after the shift from LD to UD is complete (i.e., 2 min, see Fig. 1 *a*) relatively long and disperse filaments are observed (Fig. 1 *d*). Evidently, a significant fraction of G-actin monomer dimerizes (LD) immediately upon the binding of salt. Formation of nuclei is signalled by the appearance of UD and the elongation phase is characterized by a gradually increasing UD yield as well as by the appearance of higher oligomers concomitant with a decrease in LD yield. Once the polymerization reaction has reached steady state the LD has more or less completely disappeared and the major cross-linking product is the UD together with fainter bands probably representing trimers and higher oligomers (see Gilbert and Frieden, 1983).

As documented in Fig. 2, the time required for the shift from LD to UD is critically dependent on the polymerization conditions used. At an actin concentration of 1 mg/ml the lag phase, as defined by the interim before the appearance of UD, is relatively short (i.e.,  $\leq 30$  s) with 2 mM MgCl<sub>2</sub> (Fig. 2 *a*) or with 2 mM MgCl<sub>2</sub>/50 mM KCl (Fig. 1 *a*), whereas it is relatively long (i.e., 2–10 min) with 2 mM CaCl<sub>2</sub> (Fig. 2 *b*) or 50 mM KCl (Fig. 2 *c*). This dependence of the LD to UD shift on the polymerization conditions is in good qualitative agreement with earlier studies (see Tobacman and Korn, 1983; Cooper et al., 1983*a*; Gershman et al., 1984) using established assays to monitor the length of the lag phase. Also in qualitative agreement with earlier studies (Tobacman and Korn, 1983; Cooper et al., 1983*a*; Frieden, 1983), the lag phase as defined by the LD to UD shift increases with decreasing actin concentration (see Fig. 3). This concentration dependence suggests that the formation of UD is somehow related to nucleation, and not just due to a simple activation of monomer and/or LD, which should not be concentration dependent. We have also noted that the length of the lag phase may vary slightly from preparation to preparation for a given polymerization condition and actin concentration. In addition to the lag phase concentration dependence, the yield of LD also decreases relative to monomer with decreasing actin concentration (see Fig. 3). This behavior argues against the LD being induced merely by some cross-linking effect, but indicates that the LD observed correlates with a distinct oligomeric species present in solution in the absence of 1,4-PBM.

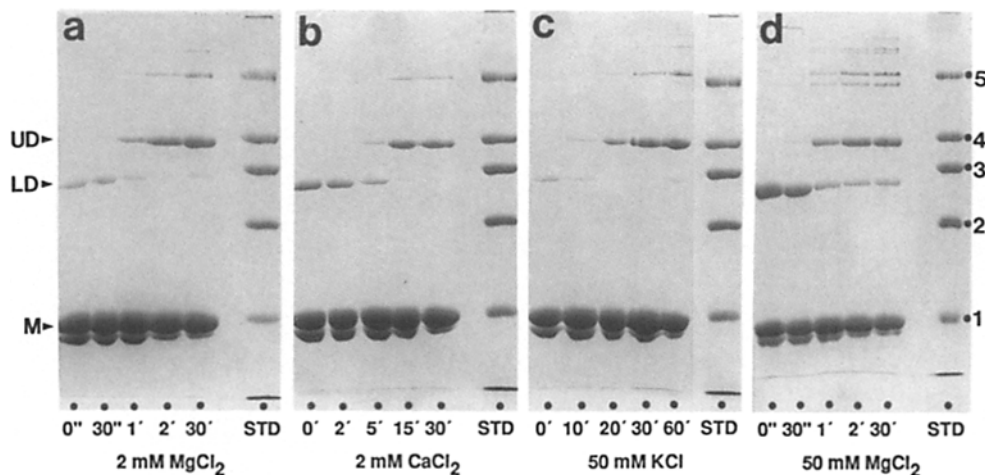
We have monitored the yield of cross-linked LD and UD



**Figure 1.** Actin polymerization as monitored by 1,4-PBM cross-linking (a), pyrene fluorescence (b), and electron microscopy (c and d). (a) Actin (1 mg/ml) was polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl. Polymerization was initiated by the addition of salt at time 0 and aliquots were removed and cross-linked with 1,4-PBM at a cross-linker/protein molar ratio of 0.5:1 at the times indicated as described in Materials and Methods. Cross-linked aliquots were analyzed by SDS-PAGE (7.5% gels). Gel standards (STD): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3);  $\beta$ -galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5). (b) Time course of pyrene-labeled actin filament formation under the same conditions as in a; 9% of the total actin was pyrenated;  $\lambda_{\text{excitation}} = 365 \text{ nm}$ ,  $\lambda_{\text{emission}} = 407 \text{ nm}$  (data from Sato et al. [1985]). (c) Electron micrograph of a negatively stained sample of an actin preparation 30 s after the initiation of polymerization under the same conditions as in a. (d) Electron micrograph of a negatively stained sample of the same actin preparation as in c but polymerized for 5 min. Bars, 100 nm.

as a function of 1,4-PBM concentration. As depicted in Fig. 4 b, if G-actin is polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl and cross-linked with varying amounts of 1,4-PBM under conditions predominantly yielding LD (i.e., immediately after the onset of polymerization), the yield of LD peaks at a 1,4-PBM/actin molar ratio of 0.5:1. This behavior is consistent with the LD cross-link occurring between residues of similar reactivity on the two actin subunits forming the LD (e.g., Cys<sub>374</sub>-Cys<sub>374</sub>; see Discussion). In contrast,

when polymerizing actin is cross-linked under conditions predominantly yielding UD (i.e., 30 min after the onset of polymerization), the UD yield increases, with increasing 1,4-PBM concentration plateauing at a molar ratio of  $\sim 2:1$  (1,4-PBM to actin) with a concomitant increase in higher molecular mass bands (see Fig. 4 c). This suggests that residues of significantly differing reactivities to 1,4-PBM are cross-linked in the UD. This was confirmed when the UD cross-link was mapped to Cys<sub>374</sub>-Lys<sub>191</sub> (Elzinga and Phelan,

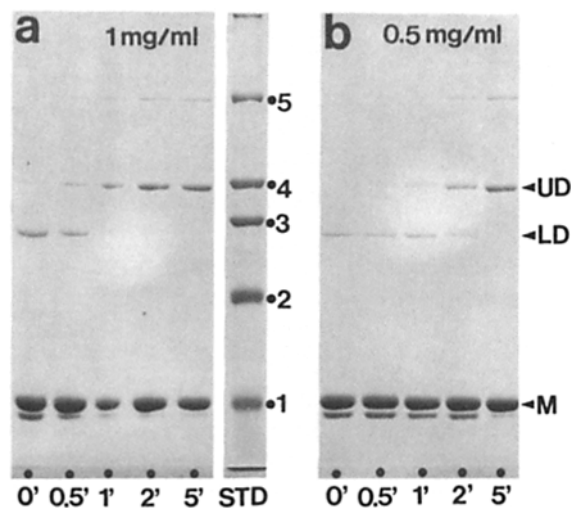


**Figure 2.** Actin polymerization as monitored by 1,4-PBM cross-linking under a variety of polymerization conditions. Actin (1 mg/ml) was polymerized with 2 mM MgCl<sub>2</sub> (a), 2 mM CaCl<sub>2</sub> (b), 50 mM KCl (c), and 50 mM MgCl<sub>2</sub> (d). At time 0 polymerization was initiated by the addition of the appropriate salt, and at the times indicated aliquots were withdrawn and cross-linked with 1,4-PBM at a cross-linker/protein molar ratio of 0.5:1 as described in Materials and Methods. Cross-linked aliquots were analyzed by SDS-PAGE (7.5% gels). Gel standards (STD): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3);  $\beta$ -galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5).

1984), as 1,4-PBM is considerably more reactive to Cys than to Lys (Knight and Offer, 1978). Under conditions where no filaments are formed (i.e., in buffer A; see Materials and Methods) there are no oligomer cross-linking bands at any cross-linker concentration tested (Fig. 4 a), indicating that the presence of LD or UD bands is not simply a cross-linking artifact, but requires the formation of distinct oligomers.

To determine the cross-linker specificity of the formation

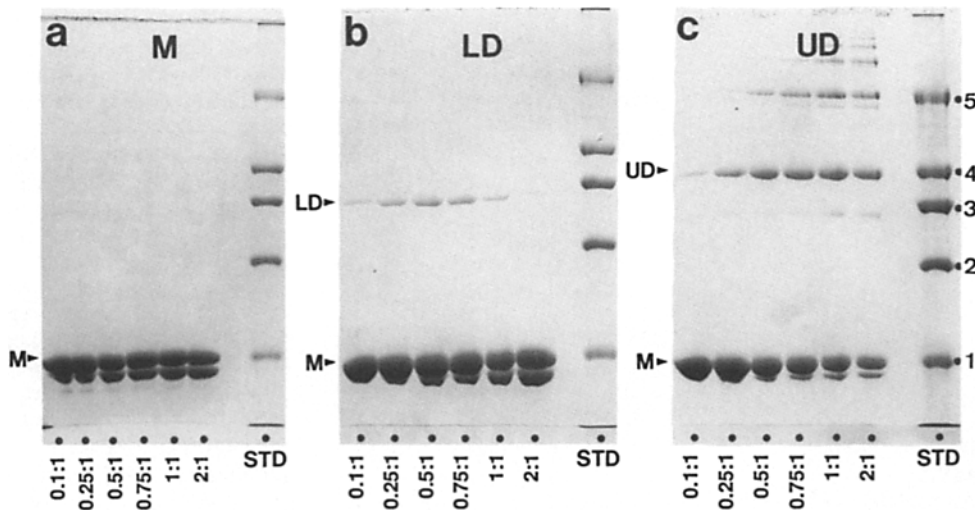
of LD and UD, we have assayed polymerization using a variety of cross-linking reagents. In the first case we have cross-linked with the ortho isomer of 1,4-PBM, 1,2-PBM. As documented in Fig. 5 when actin is polymerized with 50 mM MgCl<sub>2</sub>, or under other standard conditions (data not shown), there is virtually no difference in the cross-linking pattern when using either 1,2-PBM or 1,4-PBM. In addition, we have cross-linked with 0.25% glutaraldehyde, a crosslinker with a significantly different reactivity, and have observed a qualitatively similar shift pattern from LD to UD (data not shown). The data indicate that the formation of LD and its shift to UD are intrinsic steps of the polymerization process and are not artifactually derived from the reactivity or geometry of a specific cross-linker.



**Figure 3.** Actin polymerization as monitored by 1,4-PBM cross-linking as a function of actin concentration. Actin at a concentration of 1 mg/ml (a) or 0.5 mg/ml (b) was polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl. At time 0 polymerization was initiated by the addition of the appropriate salt, and at the times indicated aliquots were removed and cross-linked with 1,4-PBM at a molar ratio of 0.5:1 as described in Materials and Methods. Cross-linked aliquots were analyzed by SDS-PAGE (7.5% gels). Gel standards (STD): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3);  $\beta$ -galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5).

#### Isolation of Cross-linked LD and UD

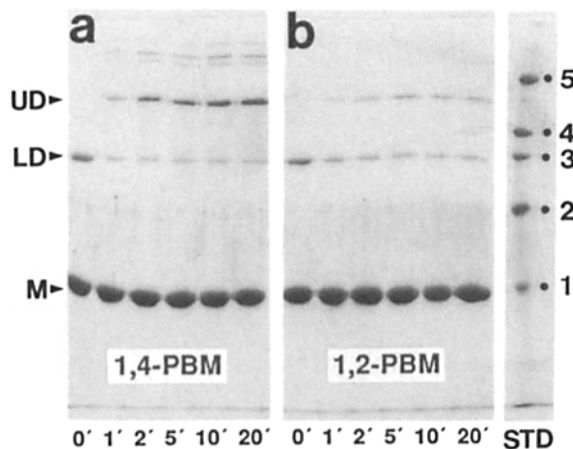
To isolate pure LD we have cross-linked 5–10 mg of actin under conditions that yield LD exclusively (i.e., immediately after the onset of polymerization with 2 mM MgCl<sub>2</sub>/50 mM KCl or with 2 mM CaCl<sub>2</sub>; see also Materials and Methods). After extensive dialysis of the cross-linked material against buffer A (see Materials and Methods), a high speed supernatant was chromatographed on a Sephadex G-200 gel filtration column to separate the cross-linked LD from monomer. The UD preparation (i.e., cross-linked 30 min after the onset of polymerization with 2 mM MgCl<sub>2</sub>/50 mM KCl; see Materials and Methods) was done in an analogous way. The corresponding column elution profiles are shown in Fig. 6 a. As can be seen in both the LD and UD preparations, a clearly defined peak is present between the void volume and the monomer peak. In fact, the LD and UD elute in the same fractions, demonstrating that, while the two dimers have different apparent mobilities on SDS-PAGE, they must have very similar Stokes radii. Accordingly, when a mixture of LD and UD was loaded onto a Sephadex G-200 column, the LD and UD coeluted in the same fractions (see Fig. 6 c). The



**Figure 4.** The effect of 1,4-PBM concentration on the monomer (*M*), LD, and UD cross-linking patterns. (*a*) *M*: actin (1.1 mg/ml) in buffer A was cross-linked with 1,4-PBM at the molar ratios indicated, and aliquots were analyzed by SDS-PAGE (7.5% gels). (*b*) *LD*: actin (1.1 mg/ml) was polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl. Immediately after the addition of salt, aliquots were cross-linked with 1,4-PBM at the molar ratios indicated and analyzed by SDS-PAGE (7.5% gels). (*c*) *UD*: actin (1.1 mg/ml) was polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl;

30 min after the addition of salt, aliquots were cross-linked at the molar ratios indicated and analyzed by SDS-PAGE (7.5% gels). Gel standards (*STD*): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3); β-galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5).

purity of the isolated cross-linked LD and UD as assayed by SDS-PAGE was typically >90% (see Fig. 6 *b*), with no cross-contamination of LD in UD preparations and vice versa. Normally, a small amount of monomer copurifies with the isolated dimers, which could be due to the copurification of some uncross-linked dimer together with the cross-linked dimeric species. Whether the LD and UD preparations are chromatographed separately or mixed before gel filtration (Fig. 6 *c*), no significant amount of contamination from higher oligomers is observed in either case.



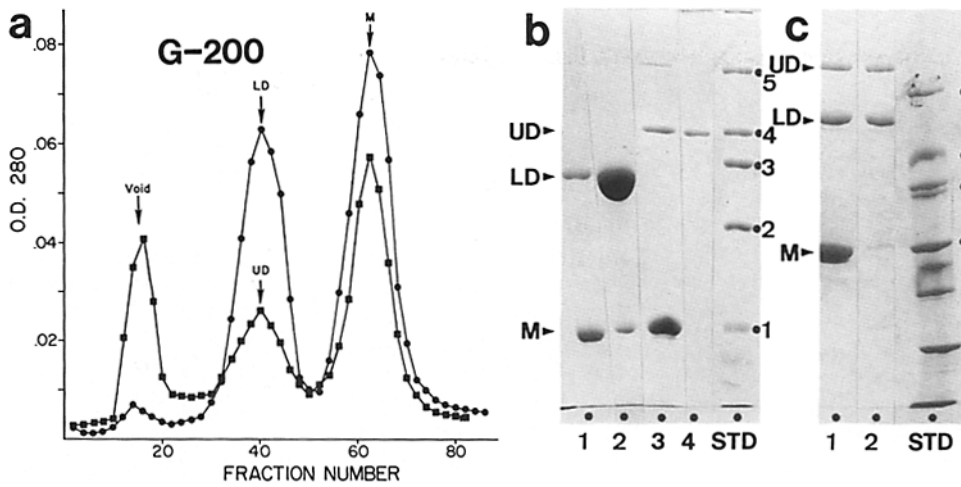
**Figure 5.** Actin polymerization as monitored by 1,2-PBM versus 1,4-PBM cross-linking. Actin (1 mg/ml) was polymerized with 50 mM MgCl<sub>2</sub>. At time 0 the salt was added to initiate polymerization, and at the times indicated aliquots were removed and cross-linked with 1,4-PBM (*a*) or with 1,2-PBM (*b*) at a molar ratio of 0.5:1 as described in Materials and Methods. Cross-linked aliquots were analyzed by SDS-PAGE (10% gels). Gel standards (*STD*): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3); β-galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5).

To further demonstrate that both cross-linked species are in fact dimers, the absolute *M<sub>r</sub>*s of isolated cross-linked LD and UD were determined by sedimentation equilibrium centrifugation (see Materials and Methods): they were measured as 89 kD for LD and 91 kD for UD (data not shown). These values are in good agreement with values determined previously for UD by Mockrin and Korn (1983). Using sedimentation velocity centrifugation we also measured the sedimentation coefficients of cross-linked LD and UD. The *s*<sub>20,w</sub> values obtained were 5.2 for LD and 4.8 for UD.

In an effort to ascertain the overall size and shape of purified LD, UD, and monomer, samples were visualized in the electron microscope by glycerol spraying and rotary metal shadowing (see Materials and Methods). While both LD (Fig. 7 *a*) and UD (Fig. 7 *b*) appear significantly larger than monomer (Fig. 7 *c*), the two dimers cannot be faithfully distinguished by this method of preparation for electron microscopy.

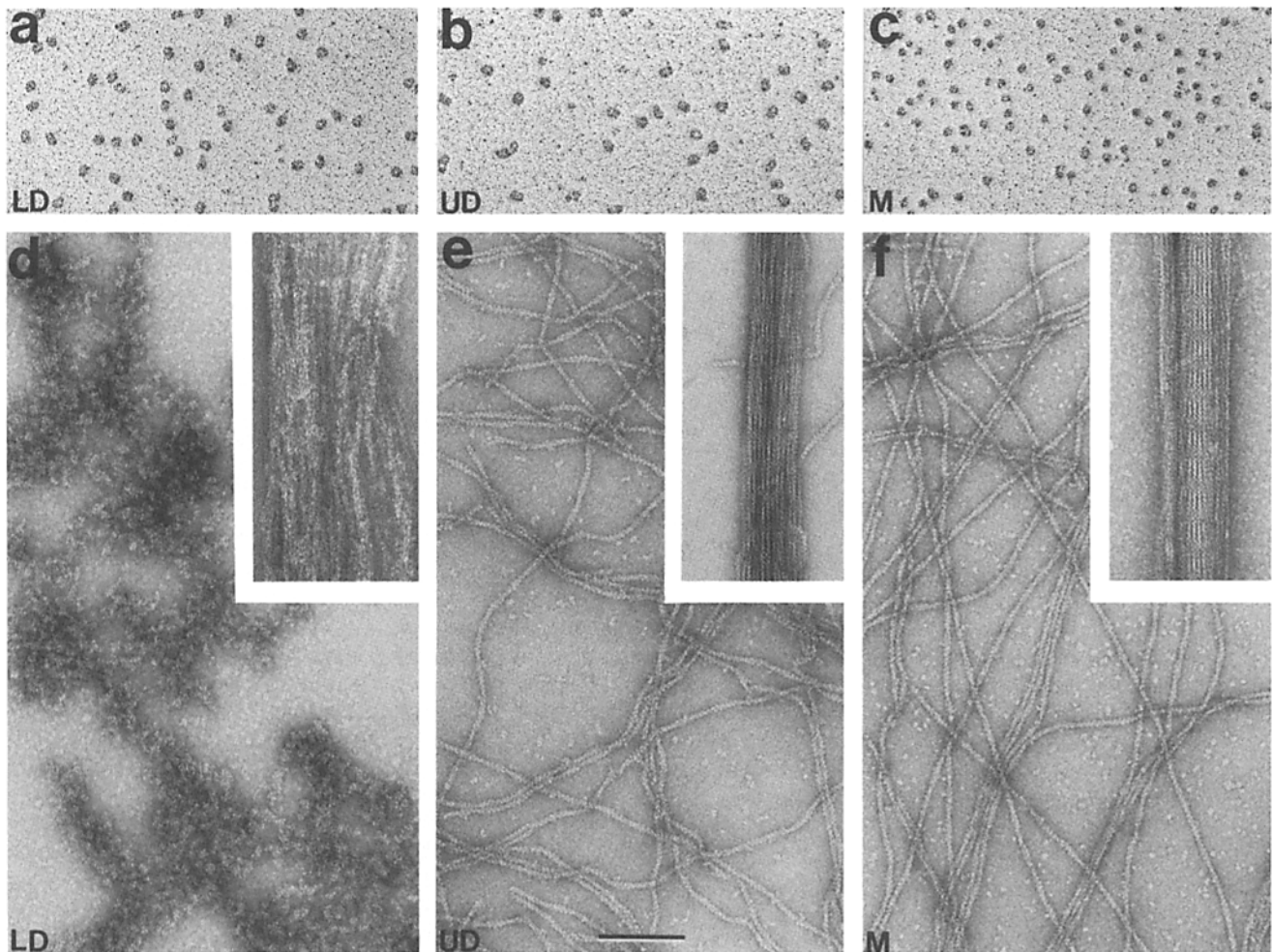
#### Assembly Properties of Cross-linked LD and UD

The ability of isolated cross-linked LD and UD to assemble into filaments was assayed by electron microscopy. Salt was added to both dimer samples to a final concentration of 2 mM MgCl<sub>2</sub>/50 mM KCl and they were allowed to polymerize at room temperature for 60 min. Under these conditions actin monomer forms long and disperse filaments (Fig. 7 *f*). When cross-linked LD is incubated under the same conditions only loose aggregates are formed (Fig. 7 *d*). However, when the purified cross-linked LD is incubated under ionic conditions (i.e., 50 mM MgCl<sub>2</sub>) that favor the formation of F-actin paracrystals with G-actin (see Fig. 7 *f*, *inset*), paracrystal-like arrays are formed (see Fig. 7 *d*, *inset*). These paracrystallike arrays, however, seem to lack some of the organization (i.e., the individual filaments appear less helical) found in paracrystals induced from unmodified G-actin. The inability of cross-linked LD to form disperse filaments while being able to form paracrystal-like bundles is consistent with



**Figure 6.** Isolation of cross-linked LD, UD, and a mixture of LD + UD by gel filtration on a Sephadex G-200 column. Cross-linked LD, UD, and a mixture of LD + UD were prepared and isolated as described in Materials and Methods. (a) The UV absorbance profile at 280 nm is shown for both LD (●) and UD (■) preparations. (b) The LD and UD peak fractions were pooled, concentrated, and subjected to electrophoresis by SDS-PAGE (7.5% gels). Lane 1, LD preparation before gel filtration; lane 2, LD pool

from G-200 column dimer peak fractions; lane 3, UD preparation before gel filtration; lane 4, UD pool from G-200 column dimer peak fractions. Gel standards (*STD*): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3);  $\beta$ -galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5). (c) The peak fractions obtained from a mixture of cross-linked LD + UD were pooled, concentrated, and subjected to electrophoresis by SDS-PAGE (10% gels). Lane 1, preparation of LD + UD mixture before gel filtration; lane 2, LD + UD pool from G-200 column dimer peak fractions. Gel standards (*STD*): rabbit skeletal muscle actin, 43 kD (1); bovine brain tubulin ( $\alpha$ - and  $\beta$ -chain), 55 kD (2); BSA, 66.2 kD (3); phosphorylase B, 92.5 kD (4).

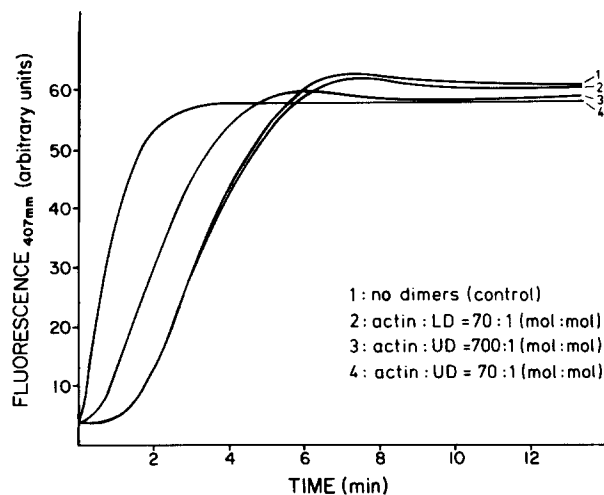


**Figure 7.** Structural and assembly properties of purified cross-linked LD and UD compared to monomer (*M*). Purified LD (a), UD (b), and *M* (c) samples are visualized in the electron microscope after glycerol spraying and rotary metal shadowing as described in Materials and Methods. To test the assembly properties of the purified cross-linked preparations, aliquots from each preparation were incubated under filament-forming conditions (i.e., 2 mM MgCl<sub>2</sub>/50 mM KCl at room temperature for 1 h). Electron micrographs of negatively stained samples are shown for LD (d), UD (e), and *M* (f). Additionally, cross-linked LD, UD, and *M* were incubated under conditions favoring the formation of paracrystalline arrays (i.e., 50 mM MgCl<sub>2</sub> at room temperature for 2 h). Electron micrographs of the negatively stained samples are shown as insets to d, e, and f. Bar, 100 nm.

the hypothesis that the LD does not define intersubunit contacts within the actin helix, but between filaments. As can be seen in Fig. 7 *e*, cross-linked UD readily assembles into filaments that are generally indistinguishable from F-actin filaments polymerized from G-actin (as seen in Fig. 7 *f*; see also Mockrin and Korn [1981]). Remarkably, isolated cross-linked UD will spontaneously assemble into filaments over a period of days in the absence of salt and ATP (i.e., in buffer A without ATP), even when kept at protein concentrations below 0.1 mg/ml and at 4°C (our unpublished observation).

### Nucleation Properties of Cross-linked LD and UD

If both types of dimers are true intermediates in the polymerization process, then purified cross-linked dimers might effect the lag phase and thus activation and/or nucleation. This was tested by monitoring the fluorescence enhancement of pyrene actin in the presence of varying amounts of cross-linked LD or UD (see Materials and Methods). As shown in Fig. 8, the presence of cross-linked LD does not effect the lag phase at all, whereas the presence of increasing amounts of UD shortens the lag phase in a concentration-dependent fashion. It should be noted that while the lag phase is shortened it is not abolished completely by the addition of cross-linked UD, indicating that the UD does not represent an "optimal" nucleus but must still undergo some change. This change might involve an activation (i.e., an internal conformational change) or a further oligomerization to a structure (i.e., a trimer) specifying all of the contacts in the actin helix (see also Mockrin and Korn, 1981; Gilbert and Frieden, 1983). Nevertheless, the effect of seeding with UD on the rate of nucleation suggests that the change concomitant with



**Figure 8.** Nucleation properties of purified cross-linked LD and UD. The fluorescence enhancement of polymerizing pyrene actin was monitored in the presence of varying amounts of purified cross-linked LD and UD. Trace 1, time course of filament formation of pyrene actin in the absence of added cross-linked LD or UD. Trace 2, time course of filament formation of pyrene actin in the presence of cross-linked LD at a molar ratio of 70:1 (monomer/LD). Trace 3, time course of filament formation in the presence of cross-linked UD at a molar ratio of 700:1 (monomer/UD). Trace 4, time course of filament formation in the presence of cross-linked UD at a molar ratio of 70:1 (monomer/UD). In all experiments 5% of the total actin was pyrenated. Actin was polymerized with 2 mM MgCl<sub>2</sub> at 25°C in all cases.  $\lambda_{\text{excitation}} = 365 \text{ nm}$ ,  $\lambda_{\text{emission}} = 407 \text{ nm}$ .

the formation of UD must play an important role in the processes that ultimately result in the formation of competent nuclei.

### Ability of Cross-linked LD and UD to Form Gd<sup>3+</sup>-induced Sheets

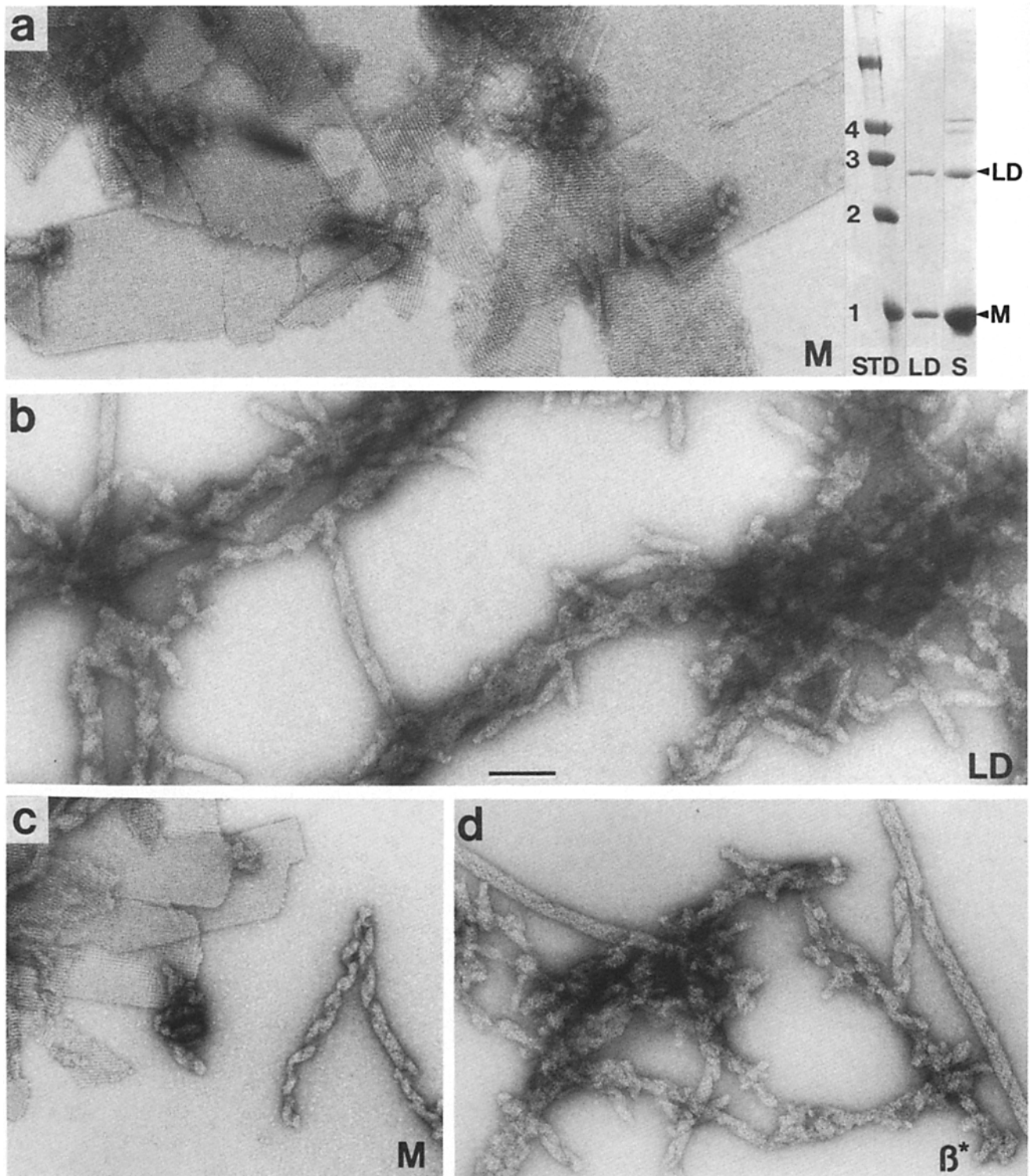
It has been shown that G-actin can be induced to form crystalline sheets in the presence of the trivalent lanthanide gadolinium (Fig. 9 *a*), and that the packing of monomers in these sheets differs significantly from that in the F-actin filament (dos Remedios and Dickens, 1978; Aebi et al., 1981; Smith et al., 1983). For this reason it was of interest to ascertain whether the cross-linked LD can be induced to form crystalline sheets. As shown in Fig. 9 *b*, when isolated cross-linked LD is incubated under sheet-forming conditions, a "folded ribbon" type structure is formed. We have observed this type of structure previously under a variety of sheet-forming conditions: it is often seen next to or continuous with normal sheets formed from native skeletal muscle actin under standard induction conditions (Fig. 9 *c*). As a result we believe that this folded ribbon type structure represents a polymorphic form, most likely a "rolled up" version of normal Gd<sup>3+</sup>-induced sheets. Moreover, when crystalline actin sheets are cross-linked, LD is the predominant cross-linked species (see Fig. 9 *a*, *inset*). These data suggest that the structural configuration of LD is more consistent with sheets than with filaments, and as the major monomer-monomer contact between actin molecules in the sheet's unit cell appears to be very similar to that between adjacent antiparallel filaments in paracrystalline arrays (see Aebi et al., 1981), this is consistent with the hypothesis that the LD is not an intrafilament dimer.

While investigating the polymerization properties of a single-site mutant  $\beta$ -actin ( $\beta^*$ -actin: Gly<sub>244</sub> to Asp<sub>244</sub> [Vanderkerckhove et al., 1980]) expressed by the transformed human fibroblast cell line HuT-14 (Leavitt and Kakunaga, 1980; Leavitt et al., 1982) we have observed the same folded ribbon type structures (Fig. 9 *d*) found with cross-linked LD (Fig. 9 *b*) under identical induction conditions. Moreover, when either  $\beta^*$ -actin or cross-linked LD are incubated under filament-forming conditions the same loose aggregates are seen (Fig. 7 *d*). It is interesting to speculate that the effect of this mutation might be to arrest filament assembly at the LD stage, thereby preventing the formation of UD and concomitant nucleation and filament formation.

### Discussion

We have assayed actin polymerization by chemical cross-linking with the bifunctional sulfhydryl reagent 1,4-PBM, and have gained new insights into the early steps by which G-actin is assembled into F-actin. As this cross-linking assay has the advantage of distinguishing monomers from dimers and higher oligomers, as well as different types of dimers (i.e., UD from LD) from each other, we have been able to show that one of the first steps leading to filament formation is the dimerization of a significant fraction of the G-actin monomer pool. In accordance with most previous studies (Wegner and Engel, 1975; Tobacman and Korn, 1983; Cooper et al., 1983*a*; Frieden and Godette, 1983; Frieden, 1983) but





**Figure 9.** Aggregation products of monomer (*M*), cross-linked LD, and  $\beta^*$ -actin induced by gadolinium ( $\text{Gd}^{3+}$ ). *M*, LD or  $\beta^*$  (0.75–1.0 mg/ml) were first dialyzed for 6 h at 4°C against precrystallization buffer (2.5 mM imidazole, 0.25 mM DTT, 0.005%  $\text{NaN}_3$ , 0.05 mM  $\text{CaCl}_2$ , pH 7.3). Next, the samples were dialyzed for 12 h at 4°C against crystallization buffer (2.5 mM Pipes, 0.25 mM DTT, 0.005%  $\text{NaN}_3$ , 25 mM KCl,  $\text{GdCl}_3$  at a molar ratio of 6:1 [ $\text{Gd}^{3+}$ /actin], pH 6.95). Negatively stained samples for electron microscopy were prepared as described in Materials and Methods. (a) Crystalline sheets obtained with normal monomeric actin (*M*). *Inset*, sheets (lane *S*) cross-linked with 1,4-PBM at a cross-linker to actin ratio of 0.5:1 as described in Materials and Methods; mixture of monomer and cross-linked LD (lane *LD*) prepared as described in Materials and Methods; gel standards (lane *STD*): ovalbumin, 45 kD (1); BSA, 66.2 kD (2); phosphorylase B, 92.5 kD (3);  $\beta$ -galactosidase, 116.25 kD (4); myosin heavy chain, 200 kD (5). All samples were subjected to electrophoresis by SDS-PAGE (7.5% gels). (b) Folded ribbons obtained with cross-linked LD. (c) Mixture of crystalline sheets and folded ribbons obtained with normal monomeric actin (*M*). (d) Folded ribbons obtained with partially purified  $\beta^*$ -actin isolated from the transformed human fibroblast cell line HuT-14 (see Leavitt et al., 1982). Bar, 100 nm.

at variance with the recently reported x-ray scattering results of Matsudaira et al. (1987), our results reveal a distinct lag between LD and filament formation for all polymerization conditions tried. The requirement for a nucleation step, as evidenced by the concentration-dependent lag phase, is further supported by recent structural advances made in our laboratory (Aebi et al., 1986; and Millonig, R., A. Engel, T. D. Pollard, and U. Aebi, manuscript in preparation). (a) Three-dimensional reconstructions of negatively stained F-actin filaments reveal two major types of intersubunit contacts: one axially along the long-pitch helices and a second between the two long-pitch helices (i.e., along the genetic helix). (b) The ability to partially unravel F-actin filaments into their two long-pitch helical strands suggests that the physically stronger intersubunit contacts are along and not between the two long-pitch helices. This type of intersubunit bonding pattern supports the nucleation model as originally envisioned by Oosawa and Asakura (1975), but differs from that of Matsudaira et al. (1987), who have suggested that actin polymerizes via a simple linear condensation mechanism. As pointed out by these authors, their type of nonnucleated assembly is more consistent with an actin filament defined by a single type of intersubunit contact (i.e., one along the genetic helix; see Egelman, 1985). However, on the basis of our structural results and the cross-linking studies presented here, we believe that the vast majority of evidence supports a nucleation-condensation mechanism where at least two types of intersubunit contacts are involved.

Several convergent lines of evidence have suggested to us that the LD is cross-linked from Cys<sub>374</sub> on the one subunit, to Cys<sub>374</sub> on the other subunit, and therefore, due to the geometrical constraints imposed by the actin helix, cannot represent a filament dimer; (i.e., a dimer along the genetic helix or one along a long-pitch helix). The Cys<sub>374</sub>-Cys<sub>374</sub> assignment is the simplest explanation for the rather complicated behavior observed when LD is cross-linked at various cross-linker concentrations (Fig. 4 b). In the first case, the observed peak in cross-linking yield at a molar ratio of 0.5:1 can be explained if both reactive residues specifying the cross-link are modified with separate cross-linkers as the 1,4-PBM concentration increases. This can only occur if the cross-link involves two residues of similar high (i.e., modifiable) affinity for the cross-linker. While it is very well documented that maleimide groups preferentially modify Cys (Cole et al., 1958; Leslie et al., 1962), it has been demonstrated that 1,4-PBM is able to cross-link Cys<sub>374</sub> on the one subunit to Lys<sub>191</sub> on the other subunit in the UD (Knight and Offer, 1978; Elzinga and Phelan, 1984). This has been explained by a cross-linking mechanism where 1,4-PBM is able to quantitatively modify the more reactive Cys and only then, when the groups are optimally oriented relative to one another, will the Lys react to form the cross-link. The UD cross-linking behavior as a function of 1,4-PBM concentration (Fig. 4 c) further supports this mechanism as the yield increases, eventually plateauing, and is not attenuated above a molar ratio of 0.5:1.

While the cross-linker concentration dependence experiments seem to argue against the involvement of a Lys residue in LD formation, this can be further tested as Lys reactivity to maleimide groups has been shown to be significantly reduced at neutral pH (Brewer and Riehm, 1967). When the cross-linking is performed at pH 7, formation of the UD

cross-link is strongly inhibited, whereas LD cross-linking is attenuated but not inhibited (data not shown). In addition to providing strong evidence against the involvement of Lys, this data also demonstrate that LD formation is not just a pH-induced artifact. As Cys<sub>374</sub> appears to be the only highly reactive Cys to maleimide derivatives under nondenaturing conditions (Elzinga and Collins, 1975; Bender et al., 1976; Lusty and Fashold, 1969), it seems that the conclusion most consistent with the available data is that the LD cross-link is between Cys<sub>374</sub> on one subunit and Cys<sub>374</sub> on the other subunit. This conclusion is further supported by cross-linking studies carried out with actin modified at this residue. (a) When we reacted actin modified with *N*-iodoacetyl-*N*-(5-sulfo-1-naphthyl)ethylenediamine or pyrene at Cys<sub>374</sub> under polymerizing conditions with 1,4-PBM, we were unable to detect any significant LD cross-linking (data not shown). (b) Sutoh (1984), using a slightly different cross-linker, also noted a dramatic attenuation of cross-linked LD yield upon Cys<sub>374</sub> modification.

As the LD cross-link appears to involve the same residue (i.e., Cys<sub>374</sub>) on the two participating subunits, the LD cannot represent a filament dimer since 1,4-PBM spans a distance of 12–13 Å (Knight and Offer, 1978), whereas the minimum distance between two of the same residues on neighboring actin subunits in the filament has to be 27.5 Å on the basis of the geometric constraints imposed by the actin helix. Definitive proof for this hypothesis, however, will have to await the rigorous chemical mapping of the LD cross-link, which is outside the scope of the present investigation.

In addition to our tentative assignment of the LD as a Cys<sub>374</sub>-Cys<sub>374</sub> cross-link, several other lines of evidence support the hypothesis that the LD does not define an intrafilament contact. The LD yield, after polymerization has reached steady state, appears to be proportional to the amount of filament bundling. That is, when actin is polymerized with 50 mM MgCl<sub>2</sub> or 50 mM CaCl<sub>2</sub> yielding F-actin paracrystals, there is much more LD at steady state (see Fig. 2 d) than when actin is polymerized with 2 mM MgCl<sub>2</sub>/50 mM KCl (see Fig. 1 a), a condition where disperse filaments predominate. In addition, we have found that cross-linked LD will not assemble into disperse filaments, but forms paracrystal-like structures (e.g., Fig. 7 d). These structures, while definitely composed of laterally aggregated strands, seem to have a less pronounced helicity than paracrystals formed from F-actin filaments (e.g., Fig. 7 f), suggesting that the LD specifies interstrand contacts, but cannot properly define the helical symmetry of the actin filament. When cross-linked LD is incubated under sheet-forming conditions, folded ribbon type structures are obtained (Fig. 9 b). We have observed structures of this type previously with unmodified actin (Fig. 9 c), and they most likely represent a polymorphic form of crystalline sheets (see Fig. 9 a). The packing of monomers in crystalline sheets has been extensively studied (Aebi et al., 1981; Smith et al., 1983), and it appears that the major intersubunit contact across the sheet dyad axis, most likely defining the LD cross-link, corresponds to a contact between adjacent filaments oriented antiparallel to each other (Aebi et al., 1981). Therefore, the ability of cross-linked LD to form some polymorphic variant of sheets is further evidence in support of an interfilament dimer. While none of these data by itself unambiguously proves that the LD defines an interfilament contact, all of the results

when taken together suggest very strongly that this is the case, and, as yet, we have no evidence in favor of the LD representing a filament dimer.

Due to the molecular symmetry of the LD, which most probably involves a dyad axis perpendicular to the filament axis when one of its monomers is oriented such that it fits into the filament (see Aebi et al., 1981; Smith et al., 1983), a direct conversion of LD to UD is very unlikely to occur, since all bonds would have to be broken and new ones established after appropriate rotations and translations of the two subunits relative to each other. A breakdown into monomer, on the other hand, may take place simply to maintain the proper monomer-polymer equilibrium during the elongation phase. Alternatively, LD, which by itself may be non-productive for nuclei formation, could directly add to nuclei or growing filaments via one of its monomers. The resulting LD-filament interaction, in turn, may destabilize the inter-subunit bond(s) holding the LD together, thereby incorporating one monomer into the growing filament and releasing the other into the monomer pool. Analogously, under polymerization conditions favoring the formation of filament bundles or paracrystals, the LD might represent a very effective building block contributing to the simultaneous elongation of two filaments with opposite polarities every time it incorporates. Whatever the exact LD consumption mechanism may be during filament formation, LD formation, which is very rapid, is not responsible for the observed lag phase. Instead the lag phase is determined by activation and/or nucleation events involving monomer and/or LD.

We have also been able to correlate the assembly properties of cross-linked LD to that of  $\beta^*$ -actin expressed by the transformed human fibroblast cell line HuT-14 (Leavitt and Kakunaga, 1980; Leavitt et al. 1982). When either the cross-linked LD or a sample enriched in  $\beta^*$ -actin are incubated under conditions that favor the formation of filaments, they both assemble into similar loose aggregates as shown in Fig. 7 *d* for cross-linked LD. Furthermore, when either cross-linked LD or  $\beta^*$ -actin are induced to form paracrystals, the same type of structures are observed again (Fig. 7 *d*, inset for cross-linked LD). Moreover, when either cross-linked LD or  $\beta^*$ -actin are incubated under conditions favoring the formation of crystalline sheets with normal actin (Fig. 9 *a*), a polymorphic variant of sheets, folded ribbons, are formed instead (Fig. 9, *b* and *d*). This behavior suggests that the mutant  $\beta$ -actin is trapped in an LD-like conformation under filament-forming conditions, thereby preventing the formation of disperse filaments but still allowing the formation of paracrystal-like arrays under appropriate salt conditions (e.g., 50 mM  $MgCl_2$ ). For this hypothesis to be rigorously tested it will be necessary to assay the assembly properties of highly purified mutant  $\beta$ -actin under various polymerization conditions. We have also been able to arrest normal actin in the LD conformation as assayed by 1,4-PBM cross-linking under some polymerization conditions (e.g., in the presence of 0.5 M  $MgCl_2$  or with 50 mM  $CaCl_2$  on ice), and the characterization of this uncross-linked LD is currently being pursued in our laboratory.

As yet, we have not systematically investigated the effect of the state of hydrolysis of the bound ATP on these phenomena, except for the fact that all experiments were performed under identical ATP conditions. Nevertheless, it is conceivable that the state of ATP hydrolysis might play an important

role in the formation of LD, as well as the role of LD in actin filament assembly, and this remains a subject for future investigation.

The data for Fig. 1 *b* were kindly provided to us by Dr. M. Sato (Sato et al., 1985). The sedimentation equilibrium centrifugation experiments were performed by Mr. A. Lustig (Biozentrum, Basel). Some of the initial experiments forming the basis for this work were done by Dr. M. Bubb, at that time a summer student in our laboratory. Mrs. R. Sütterlin provided some of the gels and micrographs for the figures. We gratefully thank Dr. J. A. Cooper for critically reading the manuscript and for making many constructive comments, and Drs. J. A. Cooper, M. Sato, and T. D. Pollard for many fruitful discussions and helpful suggestions during the course of the work.

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