Cooperative Association of Actin Protomers and Crosslinked Actin Oligomers in Filaments at Low Ionic Strength[1]

Hidetake Miyata,[2] Kazuhiko Kinosita, Jr., and Gerard Marriott[3]

Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1, Hiyoshi, Kohoku-ku, Yokohama 223

F-Actin treated with a half molar equivalent of the heterobifunctional cross-linker, 4-bromomethyl-3-nitrobenzoic acid succinimide ester (BNBA-SE), produced a population of actin oligomers containing 2-14 or more protomers and a significant amount of uncrosslinked actin protomers. The crosslinking reaction is presumed to occur between Cys 374 of one actin protomer with Lys 191 of an adjacent protomer on the genetic helix of F-actin, in a similar manner to *N*-maleimidobenzoic acid succinimidyl ester, which shares similar reactive groups and crosslinking dimensions. Crosslinked oligomers and uncrosslinked protomers were found to form filaments that sediment after high-speed centrifugation, even in a buffer of low ionic strength [G-buffer: 2 mM tris-hydroxymethylaminomethane (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.3 mM NaN_3, 5 mM 2-mercaptoethanol] where affinity between actin protomers usually becomes weak, leading to the depolymerization of native F-actin. By performing the crosslinking reaction in the presence of an environment-sensitive fluorescent label, 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan), which competes with BNBA-SE for Cys 374 of actin protomers, fluorescent, crosslinked F-actin filaments were obtained which were also stable in G-buffer. Fluorometric analysis of actin labeled with acrylodan (prodan-actin) in these depolymerization-resistant filaments suggested that the molecular environment around Cys 374 of actin protomers is similar to that of actin protomers in native F-actin in F-buffer (G-buffer plus 0.1 M KCl and 1 mM $MgCl_2$). When G-actin labeled with acrylodan was co-polymerized with non-fluorescent crosslinked F-actin, some of the labeled actin was incorporated into filaments that were resistant to depolymerization in G-buffer. The emission spectrum of the prodan-actin in these filaments measured in G-buffer was almost identical to that of prodan-actin in native F-actin in F-buffer. Our interpretation of this result is that actin protomers are locked into an F-actin-like conformation in the depolymerization-resistant filament by the subunit interactions they make with crosslinked oligomers. We also present evidence which suggests that the depolymerization rate in G-buffer of filaments made with crosslinked oligomers is much slower than that of native actin because the ends of the depolymerization-resistant filaments are capped with crosslinked oligomers.

Key words: acrylodan, actin-actin interface, cooperativity, crosslinking.

Actin is a major component of the contractile apparatus in skeletal muscle and non-muscle cells. Purified actin exists in either filamentous (F-actin), or monomeric (G-actin) form depending upon ionic strength (*1*). In the presence of salt (usually KCl and/or $MgCl_2$) G-actin polymerizes into a helical filament, which can be described either as a left-handed one-start genetic helix with a pitch of 27.5 Å and a rotation of the actin molecule of $-166.2°$ along the filament axis (*2*) or a right-handed, two-start long pitch helix. Upon reduction of the ionic strength, F-actin depolymerizes into G-actin.

Actin protomers can be crosslinked in the filament between the thiol group of Cys 374 of one protomer and the ε-amino group of Lys 191 of the adjacent protomer along the genetic helix with bifunctional crosslinking reagents such as pPDM or *N*-maleimidobenzoic acid succinimidyl ester (MBS) (*3-6*). The resultant crosslinked products contain actin monomers and an ensemble of actin oligomers (*5*). Even after the ionic strength is reduced, the products

are found to be resistant to depolymerization at low ionic strength, a condition that normally leads to a decreased interaction of the terminal actin protomer with its adjacent protomers in the filament (*7, 8*). This resistance to depolymerization at the filament ends is an interesting effect which has similarities to the effect of capping proteins and drugs that prevent depolymerization. The stability of these filaments in G-buffer also provides an opportunity to study the conformation of actin protomers trapped within the stable filament in low salt buffer. In this paper we present a description of the properties and conformation of actin protomers trapped in BNBA-SE-crosslinked actin filaments which are resistant to depolymerization at low ionic strength.

MATERIALS AND METHODS

Materials—Actin was prepared from rabbit skeletal muscle acetone powder according to the method of Spudich and Watt (*9*). BNBA-SE was synthesized according to the method of Marriott *et al.* (*10*). Acrylodan was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade. Actin concentration was estimated from the absorbance at 290 nm ($A = 0.63$ ml/mg) (*11*), and BNBA-SE concentration was calculated from the absorbance at 350 nm ($\varepsilon_{350} = 500$ M$^{-1} \cdot$cm^{-1}) (*10*). The concentration of crosslinked F-actin was calculated after correction for the absorption at 290 nm of BNBA-SE ($\varepsilon_{290} = 670$ M$^{-1} \cdot$cm^{-1}) (*10*).

Preparation of BNBA-SE-Crosslinked F-Actin—Figure 1a shows the flow-chart of the preparation of BNBA-SE-crosslinked F-actin. The crosslinking reaction was carried out in G′-buffer [2 mM Tris (pH 8.0), 0.2 mM ATP, 0.2 mM CaCl$_2$, 0.3 mM NaN$_3$] plus 0.1 M KCl, and 1 mM MgCl$_2$. Actin had been polymerized with 0.1 M KCl and 1 mM MgCl$_2$ at room temperature for 1 h before the start of the reaction. F-Actin (50–80 μM) and BNBA-SE (25–40 μM) were mixed in the reaction mixture at 1:2 molar ratio, and the reaction was allowed to proceed at 20°C for 15–30 min and terminated by addition of 10 mM 2-mercaptoethanol. The mixture was then ultracentrifuged in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA) at 350,000×g for 1 h at 4°C (k factor was 22, allowing the sedimentation of molecules with an S value $\geq$ 22), and the pellet was homogenized in G-buffer (G′-buffer plus 5 mM 2-mercaptoethanol) and then dialyzed against the same buffer to reduce the ionic strength. The dialyzed material was sonicated at 0°C for about 10 s in a bath type sonicator (Model G112SPIT, Laboratory Suppliers, Hicksville, NY), followed by another ultracentrifugation under the same conditions as above. The supernatant was saved for the analysis by SDS-PAGE and we designated it as hs-sup1. The pellet was homogenized in G-buffer, then centrifuged at a low speed (20,000×g) for 20 min at 4°C to remove any aggregated material. The supernatant of this low-speed centrifugation was again cycled through the above procedure (including two ultracentrifugation steps and the low-speed centrifugation step). The supernatant obtained from the ultracentrifugation step of the second cycle was designated as hs-sup2. The pellet fraction was resuspended in G-buffer and clarified with a 20,000×g low-speed centrifugation. This sample was used in the studies outlined in this paper and is designated XLFA. The actin concentration

in XLFA was expressed as an equivalent monomer concentration estimated from the actin absorbance at 290 nm after correction for the absorption of BNBA-SE as described above. It was assumed that the protomer absorbance did not change after the crosslinking. About two BNBA-SE molecules were found to be incorporated in one protomer in XLFA, indicating that Lys and/or Cys which were not involved in the crosslinking reaction reacted with the crosslinker. The yields of XLFA for the three preparations were 22, 58, and 33% in weight ratio.

Preparation of a Fluorescently Labeled Ensemble of Crosslinked Actin Oligomers—The concentrations of F-actin, acrylodan, and the crosslinker were 50, 25, and 25 μM, respectively. The reaction was carried out at 22°C for 2 h in F-buffer and was terminated by the addition of 10 mM 2-mercaptoethanol. The reaction mixture was then processed in the same manner as for the preparation of crosslinked actin described above (see Fig. 1a). The supernatant fraction obtained from the final high-speed ultracentrifugation step of the second cycle was designated fl-hs-sup2 and saved for later fluorescence analysis. The pellet fraction was resuspended in G-buffer and clarified with a low-speed centrifugation run. This was used in the experiments described in this paper and is designated fl-XLFA.

Labeling of Actin Monomers with Acrylodan—To investigate the interaction between the ensemble of crosslinked actin oligomers and uncrosslinked protomers, G-actin was labeled with acrylodan according to Marriott *et al.* (*12*). To estimate the labeling ratio, the absorbance of the actin-bound acrylodan ($\varepsilon_{290} = 18,500$ M$^{-1} \cdot$cm^{-1} (*12*)] was used. Hereafter, we call the labeled G-actin, P-GA.

P-GA changes its emission characteristics upon polymerization (*12*): the emission peak intensity increases by 1.9 times and the peak wavelength blueshifts about 20 nm. This feature makes the label suitable to distinguish G-actin from F-actin.

Co-Polymerization of P-GA with an Ensemble of Cross-linked FA (XLFA)—To investigate the effect of XLFA on the polymerization of P-GA, 5 μM P-GA was added to various concentrations (0.05–0.5 μM) of XLFA before the addition of 0.1 M KCl and 1 mM MgCl$_2$ to induce polymerization at 20±1°C. The polymerization was monitored using the increase of the emission intensity at 450 nm with excitation at 420 nm (*12*). The initial rate of the fluorescence increase, a measure of the polymerization, was greatly enhanced in the presence of XLFA which suggests that (i) P-GA and XLFA form filaments and (ii) XLFA acts as a nucleator in the polymerization of G-actin (*7, 8*).

For fluorometric measurements of P-GA in these filaments, 4.5 μM XLFA and various concentrations of P-GA (0.5–5 μM) were co-polymerized as described above for 1 h and the reaction mixture was ultracentrifuged (350,000× g for 1 h at 4°C). The pellet was homogenized in G-buffer and the homogenate dialyzed against G-buffer for 12 h. The dialyzed material was sonicated and again ultracentrifuged and the pellet homogenized in G-buffer. The supernatant (hs-copol-sup) was saved for later spectral analysis. The fluorescent pellet was resuspended in G-buffer and clarified by a low-speed centrifugation run (20,000× g for 20 min). This fraction, designated P-GA/XLFA, was used as the source of fluorescent actin filaments resistant to depolymerization in low salt.

The Effect of Cytochalasin B on the Nucleating Activity

of XLFA—The XLFA-dependent nucleation of actin polymerization was examined in the presence of cytochalasin B (CB). XLFA (0.5 μM) was mixed in G-buffer with 5 μM PG-A plus various concentrations of CB (1–5 μM) for 5 min at $20 \pm 1^{\circ}$C before the addition of 0.1 M KCl plus 1 mM MgCl$_2$. The polymerization rate was estimated as described above.

Measurement and Analysis of the Fluorescence Spectra—Fluorescence spectra were measured between 400 and 600 nm with excitation at 385 nm (*12*) using a spectrofluorophotometer (F-4010, Hitachi, Tokyo) at $22 \pm 2^{\circ}$C. When necessary, the difference between two spectra was obtained by digitally subtracting one spectrum from the other.

RESULTS

Analysis of the Ensemble of Crosslinked Oligomers and Their Association in Filaments at Low Ionic Strength—The crosslinking reaction produced actin oligomers crosslinked to various degrees, as demonstrated in the electrophoretogram (Fig. 1). During the processing of crosslinked F-actin in low salt, some actin oligomers as well as uncrosslinked protomers dissociated and were found in the supernatant fraction of the ultracentrifugation steps [hs-sup1 (lane 4) and hs-sup2 (lane 6)]. No significant amount of protein in XLFA was found to sediment in a low-speed centrifugation run (lane 5 vs. lane 7). Minor bands appear below the monomer and the dimer bands. These may be due to intramolecular crosslinking, as was the case with the use of MBS or pPDM as crosslinkers (*6, 7*). But it is unlikely that the existence of these species affects in any way the major conclusion drawn below, because their amount is small

compared to those of other species (dimer, trimer, *etc.*).

A significant amount of uncrosslinked protomers was found in the pellet of the second centrifugation run. The sedimentation coefficient of an uncrosslinked protomer is 3.4 S (*13*), which is too small to sediment under this centrifugation condition; this argues for the view that these protomers remain specifically associated in filaments with the crosslinked oligomers even after mechanical stress (homogenization and sonication) in G-buffer.

The ensemble of actin subunits in the hs-sup2 has a sedimentation coefficient of ≤ 22, which reflects the population of actin dissociated by sonication from the barbed or pointed end of stable filaments.

Nucleation Effect of XLFA—XLFA nucleated the polymerization of G-actin in the same manner as crosslinked actin dimers (*14*) and trimers (*15*). Figure 2a shows that the rate of polymerization of P-GA was enhanced in proportion to the concentration of XLFA. In a separate experiment, 6.7 μM XLFA was mixed in G-buffer with 0.67 μM P-GA and the emission spectrum was examined. Essentially no change in the emission spectrum was observed even after 1 h incubation. Addition of 0.1 M KCl and 1 mM MgCl$_2$ caused a blueshift and an increase in the emission peak. Thus, the presence of XLFA alone was not sufficient to polymerize P-GA.

As demonstrated in Fig. 2b, this nucleating activity was dramatically reduced upon the addition of cytochalasin B, a barbed end capper (*16*). The residual polymerization observed above 2 μM cytochalasin B was presumably due to polymerization at the pointed end (*17*).

We investigated *in vitro* motility of XLFA, which was visualized with rhodamine-phalloidin, over heavy meromy-
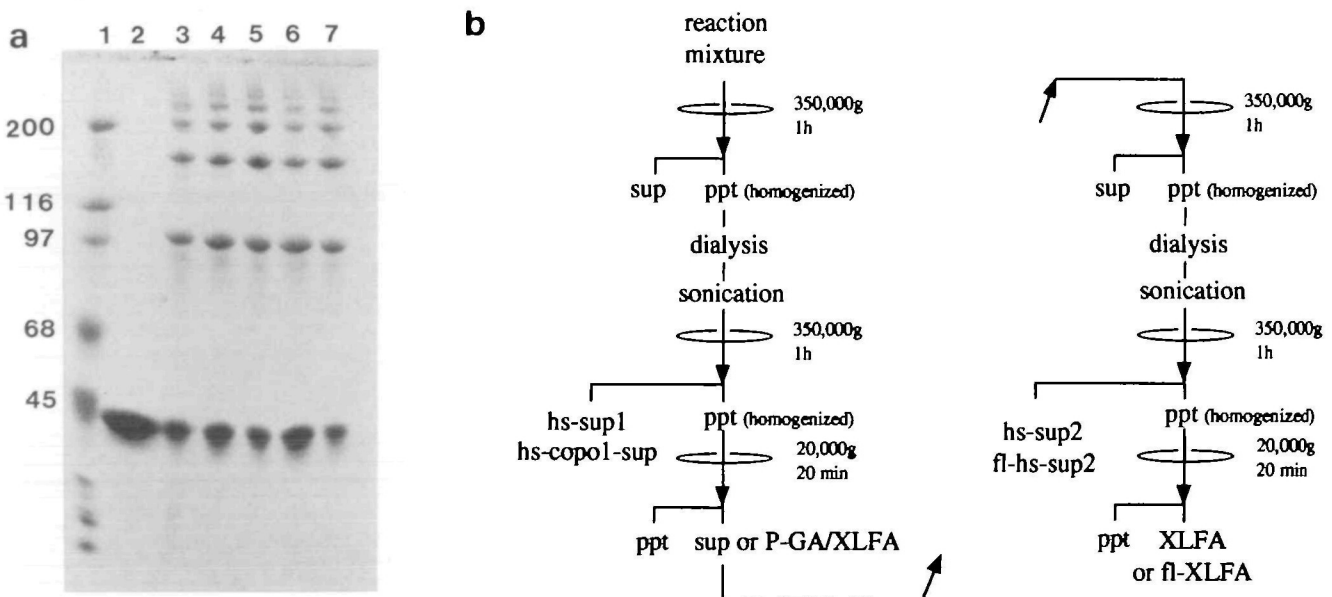
Fig. 1. Preparation of BNBA-crosslinked F-actin: analysis of the fractions by SDS PAGE and the flow chart of the preparation procedure. a, SDS-PAGE analysis. Lane 1, molecular weight standards: the numbers on the left indicate the molecular weight in kDa; lane 2, actin; lane 3, reaction mixture; lanes 4 and 5, hs-sup1 and low-speed supernatant obtained in the first cycle of the preparation; lane 6, hs-sup2, obtained in the second cycle of the preparation; lane 7, crosslinked F-actin fraction (XLFA) obtained after the second low-speed centrifugation. The total protein obtained at each step was 19.2 mg (lane 3), 3.9 mg (lane 4), 14.7 mg (lane 5), 1.1 mg (lane 6), and 12.2 mg (lane 7). In all lanes but lane 1, 5 μg of protein was loaded. For the name of the fractions, see the flow chart and the text. b, Flow chart. The left and the right columns indicate the first and the second cycles of preparation. The names of the fractions given in the text are indicated. For details of the preparation, see "MATERIALS AND METHODS."

osin bound to a glass surface. Fluorescence microscopy revealed that fluorescent filaments, which were indistinguishable from native F-actin, were sliding with speed similar to that of native F-actin (not shown). The crosslinking reaction did not seem to affect the interaction of individual actin protomers in the XLFA and heavy meromyosin.

Properties of the Fluorescent XLFA (fl-XLFA)—To investigate further the conformation of actin protomers in depolymerization-resistant crosslinked actin filaments, we prepared crosslinked actin filaments labeled in the presence of acrylodan. Figure 3a compares the emission spectra of fl-XLFA and the fl-hs-sup2, both measured in G-buffer. Also shown are the spectra of P-GA in G-buffer and P-GA in native F-actin filaments in F-buffer. For comparison, all spectra are presented with their peak heights matched. The shape of the emission spectrum of fl-XLFA measured in G-buffer was similar to that of P-GA in native F-actin in F-buffer, and not that of G-actin in G-buffer, while the emission spectrum of the fl-hs-sup2 was similar to that of P-GA in G-buffer, which is as expected, since the actin-bound label will always be in the barbed end subunit in oligomers, and consequently in oligomers that dissociate from filaments in G-buffer the probe will be exposed to the solvent as in P-GA monomers. A better match of the spectrum of P-GA in depolymerization-resistant actin (fl-XLFA) with that of P-GA in native F-actin was obtained upon subtraction of the spectrum of fl-hs-sup2 (multiplied by an appropriate numerical factor) from that of fl-XLFA, as shown in Fig. 3b.

We found that even if fl-XLFA were diluted in G-buffer to 6 nM, no red shift of the emission peak occurred, indicating that the dissociation constant for the interaction between the crosslinked oligomers and the uncrosslinked protomers was of the order of 10^{-9} M or less.

One may argue that the spectral difference between fl-XLFA and native F-actin in the longer-wavelength region is due to the existence of free labeled actin monomers in the fl-XLFA fraction. However, this possibility is unlikely, because if that happens, a shoulder should appear around 490 nm (*12*), which is not the case. At present, the reason for the residual spectral difference is not clear. The spectra are different not only between fl-XLFA and P-GA in native F-actin, but also between P-GA and the fl-hs-sup2 (Fig. 3a, dotted and dot-dash lines), which may indicate that the

crosslinker bound to site(s) other than the crosslinking sites somewhat affected the micro-environment of the actin-bound probe. With this reservation, we conclude that the emission spectra of both the labeled protomers and oligomers are quite similar to that of P-GA in native F-actin in F-buffer.

The spectral similarity suggests that the molecular environment of the probe in fl-XLFA is similar to that found for P-GA in native F-actin in F-buffer. Prodan is attached to Cys 374 of actin (*12*), which resides at or near the interface between two actin protomers adjacent along the genetic helix of F-actin (*18*). Thus, the structure in the vicinity of this residue in protomers trapped within fl-XLFA in G-buffer is similar to that of protomers in native F-actin in F-buffer.

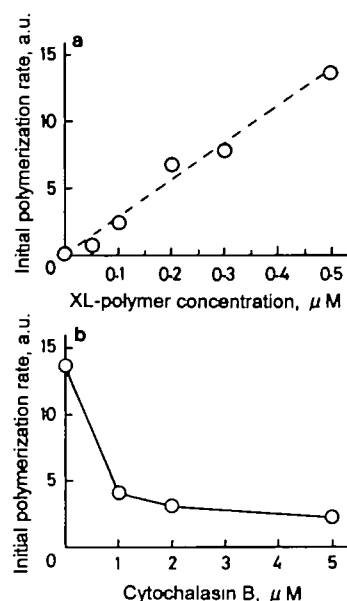*Co-Polymers of P-GA and XLFA (P-GA/XLFA)*—When P-GA and XLFA were co-polymerized and processed

Fig. 2. **The nucleation activity of crosslinked actin oligomers.** a: Relationship between the concentration of crosslinked FA (XLFA) and the initial rate of polymerization of 5 μM acrylodan-labeled actin (P-GA, the labeling ratio was 6.2%). b: Decrease of the initial polymerization rate upon the addition of cytochalasin B.
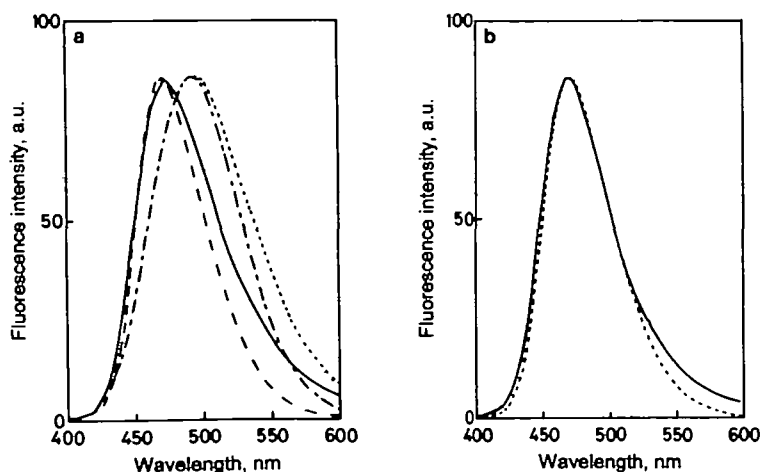

Fig. 3. **The emission spectrum of the fluorescent crosslinked F-actin (fl-XLFA).** The excitation wavelength was 385 nm. a: The spectrum of fl-XLFA (solid line), fl-hs-sup2 (dotted line), P-GA in F-actin (dashed line), and labeled G-actin (dot-dash line). The spectra were measured in G-buffer, except in the case of P-GA in F-actin when 0.1 M KCl and 1 mM MgCl₂ were present. For comparison, the peak heights of all the spectra were adjusted to be equal. Actual protein concentrations were: 1 μM for fl-XLFA and fl-hs-sup2; 4.8 μM for F- and G-actin. b: A difference emission spectrum obtained by subtracting the spectrum of fl-hs-sup2 from that of fl-XLFA (solid line). For comparison, the spectrum of P-GA in F-actin (dotted line) is also plotted.

as described in "MATERIALS AND METHODS," fluorescent co-polymers (P-GA/XLFA) were obtained that were resistant to depolymerization in G-buffer. P-GA is probably associated with these stable filaments by actin oligomers since no filament was obtained when a solution of 9.5 μM P-GA alone was processed in the same manner: *i.e.*, P-GA is completely depolymerized under the same condition.

The shape of the emission spectra measured in G-buffer of P-GA in P-GA/XLFA, prepared with 0.5 or 1.0 μM P-GA was identical to that of P-GA in native F-actin in F-buffer (Fig. 4a, two solid lines from the bottom). In the case of P-GA/XLFA prepared with 1.5 to 5.0 μM P-GA, the emission spectra were slightly red-shifted compared to P-GA in native F-actin, and this was probably due to the existence of some monomeric P-GA in these fractions: thus, when the spectrum of P-GA in G-buffer, multiplied by an appropriate numerical factor, was subtracted from each of these spectra, the resultant spectra completely overlapped with that of P-GA in native F-actin (not shown). The increase in the amount of free P-GA in filaments containing a higher ratio of monomer/oligomer compared to filaments containing a smaller ratio of monomer/oligomer might be expected because they will be more sensitive to severing induced by sonication and/or mechanical disruption (see "DISCUSSION"). The shape of the emission spectrum of P-GA in the hs-copol-sup was identical to that of monomeric P-GA in G-buffer for both concentration ranges of P-GA (not shown).

The stability of P-GA in P-GA/XLFA filaments was analyzed by recording the spectrum of P-GA before and after incubation for 40 h at 0°C. The emission spectrum of P-GA in P-GA/XLFA was only slightly red-shifted with the presence of a shoulder at 490 nm (Fig. 4a, dotted lines). Subtraction of a percentage of the pure P-GA spectrum from this spectrum resulted in a spectrum superimposable on that of P-GA in native F-actin (not shown).

This method was used to calculate the amount of P-GA in the co-polymer, which could be determined as a function of P-GA concentration in the initial co-polymerization mixture, as shown in Fig. 4b. To obtain this, we assumed that actin oligomers were fully recovered in P-GA/XLFA, and that these oligomers behave as individual units containing 30 protomers. (Using the minimum S value of 22 for the high-speed centrifugation run according to the method of

Attri *et al.* (*19*), we estimate that the number of protomers, including those in oligomers, in XLFA filaments is at least 30. Hence we assumed, for the purpose of presentation of the amount of bound P-GA in XLFA, that each XLFA consisted of 30 protomers.)

The number of bound P-GA estimated immediately after the preparation (Fig. 4b, circles) monotonously increased with the increase in P-GA concentration in the initial co-polymerization mixture. Approximately 10 P-GA molecules were bound to one XLFA unit at 5 μM P-GA. The number decreased (triangles) after the incubation of the co-polymers at 0°C for 40 h: depending upon the initial P-GA concentration, 9 to 63% of initially bound P-GA dissociated during the incubation period.

DISCUSSION

Stable Association of Crosslinked Actin Oligomers and Uncrosslinked Protomers at Low Ionic Strength—Crosslinked depolymerization-resistant filaments (XLFA) were characterized by gel electrophoresis, fluorometric analysis of P-GA and measurements of their nucleating effect on the polymerization of G-actin and its inhibition by cytochalasin B. The results suggest that even at low ionic strength, crosslinked actin filaments possessed a structure similar to that of native F-actin in F-buffer. It was deduced based on the sedimentation behavior that each XLFA filament in G-buffer consisted of 30 or more protomers and higher oligomers.

Spectrofluorometric analysis of P-GA incorporated within fl-XLFA suggests that even at the low ionic strength the labeled protomers or oligomers in the filament assume a conformation similar to that of protomers in native F-actin (F-like conformation) in F-buffer.

The mechanism by which the F-like conformation was stabilized in G-buffer probably resides in the crosslinking of adjacent protomers. Lys 191 is a potential target of BNBA, which is similar in size ($\sim$1.2 nm) to pPDM and MBS (1.2-1.4 nm) (*5, 6*). Since Lys 191 and Cys 374 are at or near the interface of two adjacent protomers along the genetic helix of F-actin, the crosslinking reaction perhaps stabilizes the subunit contact between two protomers, which are then unable to change their conformation from F-like to that of G-actin even when the ionic strength is reduced: *i.e.*, the
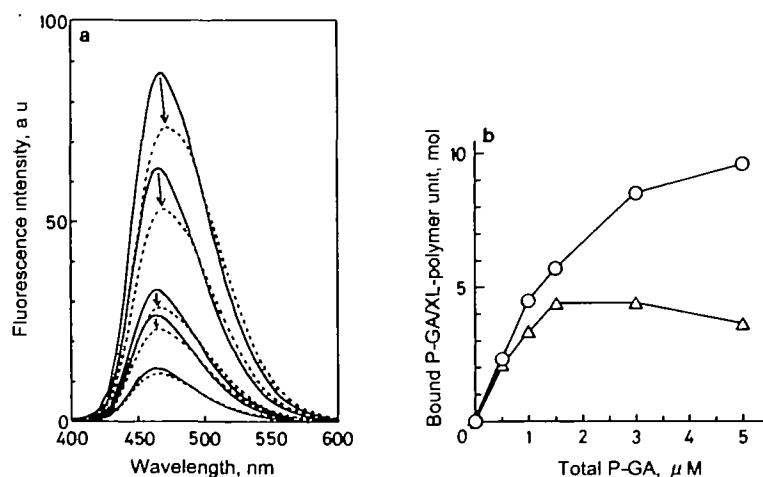

Fig. 4. **Fluorometric analysis of the P-GA/XLFA fractions.** a: The emission spectra of P-GA in P-GA/XLFA fractions prepared with 4.5 μM XLFA and various concentrations of P-GA. Solid lines, the P-GA/XLFA spectra measured immediately after the preparation. The P-GA concentrations were 0.5, 1.0, 1.5, 3.0, and 5.0 μM from the top to the bottom curve; dotted lines, the P-GA/XLFA spectra measured after the 40 h incubation at 0°C. The direction of the change in each spectrum is denoted with downward arrows. b: Number of P-GA molecules incorporated in the P-GA/XLFA as a function of the initial P-GA concentration, obtained immediately (circles) and 40 h (triangles) after the P-GA/XLFA preparation.

crosslinked oligomers force the protomers trapped within XLFA to assume an F-like conformation.

This locked, F-like conformation, which we believe can only exist in filaments capped with oligomers, presumably allows the protomers to contact each other through three inter-protomer or inter-oligomer contacts, as does a protomer in native F-actin (a protomer at the end of an XLFA will make only 2 contacts and will dissociate like protomers in native F-actin in low ionic strength buffer, see below). This is consistent with the experimentally-derived notion that XLFA possess a structural similarity to F-actin and explains the stability of these filaments in low salt buffer, since three contacts must be broken simultaneously to liberate an oligomer unit from the end of XLFA, as schematized in Fig. 5a. These contacts must be as stable as those in F-actin, for which the dissociation constant has been calculated to be 10^{-13} M (20).

Uncrosslinked protomers are unlikely to be present at the ends of stable, crosslinked actin filaments, since they would be attached by only two bonds (Fig. 5b, arrow). End-associated protomers in normal F-actin have an association constant of about 10^5 M^{-1} (20), which is too small to explain the stable association of the uncrosslinked protomers in XLFA in G-buffer. Based on this consideration, as schematized in Fig. 5b (arrowhead), the protomers in XLFA must be sandwiched between crosslinked oligomers which also cap filament ends.

Association between P-GA and XLFA at Low Ionic Strength—The similarity of the emission spectra of P-GA in the co-polymer (P-GA/XLFA) measured in G-buffer and P-GA in F-actin in F-buffer indicates that at low ionic strength the conformation of P-GA in P-GA/XLFA is similar to that of P-GA protomers in native F-actin. Exactly how P-GA and XLFA associate together in P-GA/XLFA cannot be readily deduced from the present data alone; however, we assume formation of block co-polymers, as schematized in Fig. 5c. Since XLFA probably acts as units of at least 30 protomers and oligomers, their constituents, the crosslinked oligomers and the uncrosslinked protomers, cannot be randomized with P-GA during the co-polymerization reaction. We further assume that the stability of the co-polymer in G-buffer was brought about by locking of the conformation of P-GA in an F-like state by crosslinked actin oligomers.

In the preparation of stable actin filaments from monomeric P-GA and XLFA, we believe P-GA polymerized between the stable units of XLFA and was incorporated within these stable filament units (Fig. 5c). This assumption arises from the observation that even in the absence of mechanical stress in G-buffer, a slow release of P-GA occurred from P-GA/XLFA, probably by random fragmentation; such a release does not easily occur in fl-XLFA where the labeled protomers are incorporated within the stable filaments.

One previous report describes a delayed depolymerization at a low ionic strength of actin filaments with a sub-stoichiometric concentration of myosin subfragment-1 bound to them (21). The time constant of the process is of the order of a few hours. The number of actin molecules stabilized has been estimated to be 5.5 for each subfragment-1 molecule. It is conceivable that the protomer conformation stabilized by subfragment-1 and the locked, F-like conformation assumed above are similar. We note,
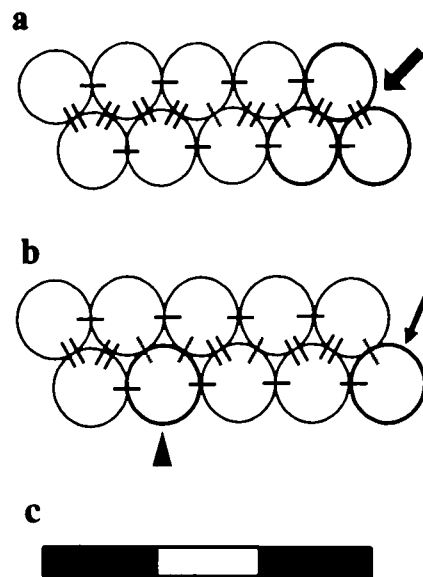
Fig. 5. **Schematic representations of inter-molecular bonds in part of a stable, crosslinked actin filament.** An arrangement of the protomers similar to that in native F-actin is assumed (see text). For the sake of presentation, part of the unit is untwisted to show the contacts between the constituents (uncrosslinked protomers and crosslinked oligomers). Double bars, the crosslinks by BNBA-SE; single solid bars, non-covalent contacts between the uncrosslinked protomers adjacent along the two-start helix; single dotted bars, non-covalent contacts along the genetic helix. a: A trimer held by three non-covalent contacts at the right end of the crosslinked actin filament (bold arrow). b: A protomer held by two bonds at the end of the unit (thin arrow), and another one (arrowhead) sandwiched between a dimer and a trimer by four bonds, indicating more stable association than the end-associated protomer. For easier identification, those indicated with arrows are also represented with bolder lines. c: Highly schematic representation of a block co-polymer made from two stable, crosslinked actin filament units (closed box) and a stretch of P-GA (open box). The length of the box does not necessarily indicate the number of actin protomers.

however, that while subfragment-1 could induce actin polymerization at low ionic strength (22), the crosslinked polymers could not, as described above.

The cooperative nature of the interaction between actin molecules seems to play an important role in the stabilization of crosslinked actin filaments in G-buffer. It has been shown that other proteins and drugs can alter or promote cooperative interactions between actin protomers in the filament; these include myosin subfragment-1 (21, 23, 24), proteolytical digestion (25) or phalloidin binding (26), gelsolin binding (27) and chemical modification (23, 26). Our study suggests that conformationally stabilized actin oligomers can also cause a cooperative conformational change that is transmitted through the actin filament. We speculate that the crosslinking reduces structural flexibility in the vicinity of the C-terminus of actin, which has been suggested to be an important factor determining the strength of the contact between the protomers (25). Such reduced flexibility would be transmitted to the uncrosslinked protomers by a mode of cooperativity based on some structural connectivity between the C-terminus and other region(s) within the molecule (28), thereby increasing the filament stability.

REFERENCES

1. Korn, E.D., Carlier, M.-F., and Pantaloni, D. (1987) Actin polymerization and ATP hydrolysis. *Science* **238**, 638–644
2. Egelman, E.H. (1985) The structure of F-actin. *J. Muscle Res. Cell Motil.* **6**, 129–151
3. Elzinga, M. and Phelan, J.J. (1984) F-actin is intermolecularly crosslinked by *N,N'-p*-phenylenedimaleimide through lysine-191 and cysteine-374. *Proc. Natl. Acad. Sci. USA* **81**, 6599–6602
4. Elzinga, M. and Hegyi, G. (1991) Crosslinking of actin with *N*-(4-azidobenzoyl)putrescine: a test of the F-actin model. *Biophys. J.* **59**, 52a
5. Knight, P. and Offer, G. (1978) *p-NN'*-phenylenebismaleimide, a specific cross-linking agent for F-actin. *Biochem. J.* **175**, 1023–1032
6. Sutoh, K. (1984) Actin-actin and actin-deoxyribonuclease I contact sites in the actin sequence. *Biochemistry* **23**, 1942–1946
7. Mockrin, S.C. and Korn, E.D. (1981) Isolation and characterization of covalently cross-linked actin dimer. *J. Biol. Chem.* **256**, 8228–8233
8. Tobacman, L.S. and Korn, E.D. (1983) The kinetics of actin nucleation and polymerization. *J. Biol. Chem.* **258**, 3207–3214
9. Spudich, J.A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **246**, 4866–4871
10. Marriott, G., Miyata, H., and Kinosita, K., Jr. (1992) Photomodulation of the nucleating activity of a photocleavable crosslinked actin dimer. *Biochem. Int.* **26**, 943–951
11. Houk, T.W., Jr., and Ue, K. (1974) The measurement of actin concentration in solution: a comparison of methods. *Anal. Biochem.* **62**, 66–74
12. Marriott, G., Zechel, K., and Jovin, T.M. (1988) Spectroscopic and functional characterization of an environmentally sensitive fluorescent actin conjugate. *Biochemistry* **27**, 6214–6220
13. Mihashi, K. (1964) Molecular characteristics of G-ADP actin. *Arch. Biochem. Biophys.* **107**, 441–448
14. Mockrin, S.C. and Korn, E.D. (1983) Kinetics of polymerization and ATP hydrolysis by covalently cross-linked actin dimer. *J. Biol. Chem.* **258**, 3215–3221
15. Lal, A.A., Korn, E.D., and Brenner, S.L. (1984) Rate constants for actin polymerization in ATP determined using cross-linked

actin trimers as nuclei. *J. Biol. Chem.* **259**, 8794–8800
16. Cooper, J.A. (1987) Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473–1478
17. Bonder, E.M. and Mooseker, M.S. (1986) Cytochalasin B slows but does not prevent monomer addition at the barbed end of the actin filament. *J. Cell Biol.* **102**, 282–288
18. Holmes, K.C., Popp, D., Gebhard, W., and Kabsch, W. (1990) Atomic model of the actin filament. *Nature* **347**, 44–49
19. Attri, A.K., Lewis, M.S., and Korn, E.D. (1991) The formation of actin oligomers studied by analytical ultracentrifugation. *J. Biol. Chem.* **266**, 6815–6824
20. Erickson, H.P. (1989) Co-operativity in protein-protein association. The structure and stability of the actin filament. *J. Mol. Biol.* **206**, 465–474
21. Grazi, E., Magri, E., and Rizzieri, L. (1989) The influence of substoichiometric concentrations of myosin subfragment 1 on the state of aggregation of actin under depolymerizing conditions. *Eur. J. Biochem.* **182**, 277–282
22. Chaussepied, P. and Kasprzak, A.A. (1989) Isolation and characterization of the G-actin-myosin head complex. *Nature* **342**, 950–953
23. Prochniewicz, E., Katayama, E., Yanagida, T., and Thomas, D.D. (1993) Cooperativity in F-actin: chemical modifications of actin monomers affect the functional interactions of myosin with unmodified monomers in the same actin filament. *Biophys. J.* **65**, 113–123
24. Ando, T. (1989) Propagation of acto-S-1 ATPase reaction-coupled conformational change in actin along the filament. *J. Biochem.* **105**, 818–822
25. O'Donoghue, S.I., Miki, M., and dos Remedios, C.G. (1992) Removing the two C-terminal residues of actin affects the filament structure. *Arch. Biochem. Biophys.* **293**, 110–116
26. Drewers, G. and Faulstich, H. (1993) Cooperative effects on filament stability in actin modified at the C-terminus by substitution or truncation. *Eur. J. Biochem.* **212**, 247–253
27. Orlova, A., Prochniewicz, E., and Egelman, E.H. (1995) Structural dynamics of F-actin: II. Cooperativity in structural transitions. *J. Mol. Biol.* **245**, 598–607
28. Crosbie, R.H., Miller, C., Cheung, P., Goodnight, T., Muhlrad, A., and Reisler, E. (1994) Structural connectivity in actin: effect of C-terminal modifications on the properties of actin. *Biophys. J.* **67**, 1957–1964