Isolation and Characterization of Covalently Cross-linked Actin Dimer*

(Received for publication, February 2, 1981, and in revised form, May 6, 1981)

Stephen C. Mockrin and Edward D. Korn

From the Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Covalently cross-linked actin dimer was isolated from rabbit skeletal muscle F-actin reacted with phenylenebismaleimide (Knight, P., and Offer, G. (1978) Biochem. J. 175, 1023-1032). The UV spectrum of the purified cross-linked actin dimer, in a nonpolymerizing buffer, was very similar to that of native F-actin and not to the spectrum of G-actin. Cross-linked actin dimer polymerized to filaments that were indistinguishable in the electron microscope from F-actin made from native G-actin and that were similar to native F-actin in their ability to activate the Mg**ATPase of myosin subfragment-I. The critical concentrations of polymerization of cross-linked actin dimer in 0.5 mM and 2.0 mM MgCl₂, 2 to 4 μM, and 1 to 2 μM, respectively, were similar to the values for native G-actin. Cross-linked actin dimer contained 2 mol of bound nucleotide/mol of dimer. One bound nucleotide exchanged with ATP in solution with a t½ of 55 min and with ADP with a t½ of 5 h. The second bound nucleotide exchanged much more slowly. The more rapidly exchangeable site contained 10 to 15% bound ADP-P, and 85 to 90% bound ATP while the second site contained much less, if any, bound ADP-P. Cross-linked actin dimer had an ATPase activity in 0.5 mM MgCl₂ that was 7 times greater than the ATPase activity of native G-actin and that was also stimulated by cytochalasin D. These data are discussed in relation to the possible role of ATP in actin polymerization and function with the speculation that the cross-linked actin dimer may serve simultaneously as a useful model for each of the two different ends of native F-actin.

Monomeric actin-ATP polymerizes into a double-stranded helical filament containing hundreds of protomers of actin. ADP with the formation of approximately 1 mol of P, for every mole of actin protomer converted from monomer to polymer. In the early model of Oosawa and Kasai (1), hydrolysis of ATP was assumed to accompany the addition of actin monomers to filament ends. Recently, Brenner and Korn (9) demonstrated the slow hydrolysis of ATP by actin below its critical concentration in 0.5 mM MgCl₂ apparently independent of any actin-actin interactions. The existence of this reaction means that monomeric actin-ADP-P, must be present, under polymerizing conditions, in solutions containing ATP. Based on more indirect evidence, Brenner and Korn (9) further proposed that filament ends might also contain an actin-ADP-P, species and that the P, produced during polymerization might be released from these terminal F-actin subunits rather than from the monomeric actin being added to the filament. Moreover, under some conditions the rate of ATP hydrolysis was less than 50% the rate of incorporation of actin protomers into actin filaments suggesting that there might be appreciable actin-ATP or actin-ADP-P, protomers within the filaments and not just at filament ends.

We thought it might be quite helpful to the understanding of these and other important aspects of actin polymerization to study the properties of a stable preparation of pure, polymerization-competent actin dimer, especially if the dimer had a conformation similar to that of F-actin protomers rather than the conformation of G-actin monomers. Such a molecule, for example, might be a useful model of one or both ends of an actin filament and allow analyses of ends that are impossible with filaments because of the very low concentration of ends relative to internal subunits. Also, such a molecule might be expected to polymerize by a simple, single reaction rather than the essentially inseparable two-step process of nucleation and elongation (1) observed with monomers.

Knight and Offer (10) showed that the bifunctional reagent phenylenebismaleimide converts F-actin into a filament containing a mixture of modified monomer protomers, cross-linked dimers, and cross-linked higher oligomers which retains the properties of the unmodified filament (11). Grumet and Lin (12) demonstrated that a crude preparation of cross-linked dimer isolated from cross-linked F-actin can nucleate the polymerization of unmodified actin monomer. In this paper, we describe some of the important properties of highly purified, covalently cross-linked actin dimer isolated from F-actin similarly reacted with phenylenebismaleimide.

EXPERIMENTAL PROCEDURES

Materials—N,N'-p-Phenylenesmaleimide (Aldrich) was recrystallized from dimethylformamide, [α-32P]ATP and [γ-32P]ATP (both 2 to 10 Ci/mmol) from New England Nuclear were diluted immediately upon arrival to 0.2 μCi/ml in 2 mM ATP and stored at -20 °C. [*32P]ADP (40 to 60 Ci/mmol, 50 μCi/ml in 2% ethanol) from Amersham was stored at -20 °C and diluted into 2 mM ADP immediately before use. The exact concentrations of the nucleotide solutions were determined from their absorbances at 259 nm after dilution into 20 mM Tris/chloride, pH 7.5, using an absorbance coefficient of 15.4 × 10² M⁻¹ cm⁻¹. The radiochemical purity of the nucleotides was determined following separation by co-chromatography with radioactive ATP, ADP, and AMP on PEI-cellulose thin layer sheets (Baker) with 0.75
M potassium phosphate, pH 3.5, as solvent. The nucleotide spots were located under UV light, excised, and counted. The radiochemical purity of \( [\gamma-^32P]ATP \) was also measured by the per cent of radioactivity released by the Ca\(^2+\)-ATPase activity of rabbit skeletal muscle subfragment-1 (13). The observed radiochemical purities were: \( \gamma-^32P \)ATP, 85 to 95%; \( \alpha-^32P \)ATP, 86 to 92%; \( ^32P \)ADP, 97%. The experimental data were interpreted accordingly.

Muscle G-actin was prepared from acetone powders of rabbit back and leg muscles by the method of Spudich and Watt (14) modified by Eisenberg and Kielley (15). \[^{[35}S\] \)Actin was isolated by the procedure of Gordon et al. (16) from Acanthamoeba castellanii grown in the presence of \[^{[35}S\] \)methionine. The concentration of monomeric G-actin was determined from its absorbance at 290 nm using an absorbance coefficient of 0.62 mg\(^{-1}\) cm\(^{-1}\) (17).

**Critical Concentrations**—Preparations of polymerized actin were centrifuged in a Beckman Airfuge for 30 min at 30 P.S.I. to remove all filamentous. The concentration of unpolymerized actin in the supernatant solution was measured by the procedure of Lowry et al. (18).

**Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—The electrophoretic procedure of Laemmli (19) was used and the 7.5% polyacrylamide slab gels were stained with Coomassie blue according to the method of Fairbanks et al. (20). Gels were scanned at 600 nm with a Quick-Scan densitometer (Helena Laboratories) and the areas under the peaks were quantified by weighing them.

**Acto-subfragment-1 ATPase**—Activation of muscle myosin subfragment-1 ATPase activity by polymerized actin monomer and polymerized cross-linked actin dimer was measured under the following conditions: 10 mM imidazole-chloride, pH 7.0, 2 mM MgCl\(_2\), 1 mM \( [\gamma-^32P] \)ATP, 4.0 \( \mu \)g of subfragment-1/ml, and polymerized actin at concentrations of 10, 20, and 30 \( \mu \)g/ml. Release of \( ^32P \) was measured by the procedure of Pollard and Korn (13). Actin monomer and dimer were polymerized for these experiments, at concentrations of 0.57 mg/ml in buffer containing 2 mM MgCl\(_2\).

**Preparation of Cross-linked Actin Dimer**—Unless otherwise noted, all steps were carried out at 4 °C. F-actin (7 mg/ml in 40 ml of 4 mM imidazole-chloride, pH 7.5, 2 mM MgCl\(_2\)) was mixed with 40 ml of 5 mM sodium borate, pH 9.3, containing 0.2 mM KCl and 0.4 mM ATP, as described by Grumet and Lin (12). Phenylenebismaleimide (0.8 ml, 2 mM in dimethylformamide) was added to the solution of F-actin (2.4 mol of phenylenebismaleimide/mol of actin protomer). The mixture was incubated for 8 min at 25 °C and the reaction was stopped by the addition of a 1.5-fold molar excess of \( \beta \)-mercaptoethanol relative to maleimide groups, according to the method of Knight and Offer (10). The cross-linked F-actin was concentrated by centrifugation at 100,000 X g for 3.5 h, and the pellet was homogenized into 40 ml of depolymerizing buffer (buffer G, 5 mM Tris-chloride, pH 7.5, 0.2 mM ATP, 0.1 mM CaCl\(_2\), and 0.01% sodium azide) and dialyzed for 72 h against two changes of 2 liters of buffer G. After the first 24 h of dialysis, the actin was sonified for two 15-s intervals at a setting of 3.5 on a Branson sonifier equipped with a microtip. Actin that did not depolymerize was removed by centrifugation of 30,000 \( \times \) g for 3.5 h and the supernatant solution was applied to a column of Sephadex G-200 (5 \( \times \) 100 cm) equilibrated with buffer G and pumped against gravity at 40 ml/h. The column eluate was monitored by \( A_{280} \) and appropriate 5-ml fractions were analyzed by dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions enriched in dimer were pooled, with minimal contamination by monomer, and concentrated to 7 ml by vacuum dialysis. The actin was polymerized by adding MgCl\(_2\) to 2 mM and incubating at 25 °C for 30 min. The polymerized actin was collected by centrifugation at 200,000 \( \times \) g for 3 h at 20 °C, homogenized in buffer G, and depolymerized by dialysis for 48 h against one change of 1 liter of buffer G. Actin that did not depolymerize was removed by centrifugation of 200,000 \( \times \) g for 3.5 h and the supernatant solution was applied to a second column of Sephadex G-200 (2.5 \( \times \) 100 cm) equilibrated with buffer G and pumped against gravity at a flow rate of 20 ml/h. The fractions containing dimer were pooled, concentrated by vacuum dialysis, and put through one more cycle of polymerization and depolymerization. Because the presence of the phenyl group affected the UV absorbance of the cross-linked actin dimer, its concentration was determined by the procedure of Lowry et al. (18) using muscle G-actin as standard.

**RESULTS**

**Purification of Cross-linked Actin Dimer**—The purity of the initial F-actin and the composition of the unfractionated reaction mixture containing cross-linked F-actin are shown in Fig. 1, lanes A and B, respectively. Upon centrifugation of the reaction mixture, all of the cross-linked actin was pelleted and only monomeric actin remained in the supernatant solution (Fig. 1, lane C). The fraction of cross-linked F-actin that depolymerized in buffer G was greatly enriched in cross-linked dimer (Fig. 1, lane E) but more than 50% of the cross-linked dimer was lost in the nondepolymerizable material that contained most of the cross-linked higher oligomers (Fig. 1, lane D). After separation on the first Sephadex G-200 column (Fig. 2), the fractions that contain cross-linked actin dimer were essentially free of higher oligomers, although they did contain some actin monomer (Fig. 1, lanes F to K). After concentration and one cycle of polymerization and depolymerization, the preparation contained about 1% trimer, 92% dimer, and 7% monomer (Fig. 1, lane Q). The final preparations contained 95 to 98% cross-linked actin dimer and only 1 to 2% monomer, by weight, as quantified by scans of gels stained with Coomassie blue (Fig. 1, lane R).

Apparently, two different forms of dimer, with different electrophoretic mobilities, are obtained in the approximate ratio of 7:1 (84% and 14%, respectively, of the final product in Fig. 1, lane R). Both forms of dimer are competent to polymerize and their ratio is unchanged throughout the purification. Although other explanations are possible, it seems likely that the two components differ in electrophoretic mobilities because of different extents of unfolding as a consequence of different intramolecular or intermolecular cross-links (each dimer will have reacted with 2 molecules of phenylenebismaleimide, only one of which is necessary to form cross-linked dimer). A similar pair of derivatized monomers can be seen in Fig. 1.

About 5 to 6 mg of cross-linked actin dimer was obtained from 275 mg of F-actin. The yield was low for several, mostly unavoidable, reasons. The maximal yield of dimer in the cross-linking reaction is only 27% (10), much of the cross-linked F-actin does not depolymerize (perhaps because of the presence of cross-linked higher oligomers), narrow fractions are pooled and the two components differ in electrophoretic mobilities because of different extents of unfolding as a consequence of different intramolecular or intermolecular cross-links (each dimer will have reacted with 2 molecules of phenylenebismaleimide, only one of which is necessary to form cross-linked dimer). A similar pair of derivatized monomers can be seen in Fig. 1.

![Fig. 1](image-url)
from the Sephadex columns to maximize purity, and vacuum dialysis and one cycle of polymerization and depolymerization entails a 30 to 30% loss of protein, in part because of the critical concentration of the cross-linked dimer.

Polymerization of Cross-linked Actin Dimer—The critical concentrations of several preparations of cross-linked actin dimer were 2 to 4 μM in 0.5 mM MgCl₂ in buffer G and 1 to 2 μM in 2 mM MgCl₂ in buffer G. These values are indistinguishable from the critical concentrations of native G-actin as measured by the centrifugation method used in this study for the dimer and as measured previously by viscosity measurements (6).

The filaments made from cross-linked actin dimer could not be distinguished by negative staining electron microscopy from filaments made from native actin monomer (data not shown). Under conditions in which the activation of myosin subfragment-1 Mg²⁺-ATPase activity is linear with F-actin concentration, polymerized cross-linked dimer and polymerized native monomer had specific activities of 23 ± 4 and 27 ± 5 μmol·min⁻¹·mg⁻¹ subfragment-1 mg⁻¹ F-actin, respectively.

UV Absorption Spectrum of Cross-linked Actin Dimer—The difference spectrum between native F-actin and monomeric G-actin has a characteristic major peak, usually recorded at 232 nm, and several minor peaks between about 270 and 290 nm (21, 22). We observed essentially the same difference spectrum between unpolymerized cross-linked actin dimer and monomeric actin as between native F-actin and G-actin (Fig. 3) except that the major peak was appreciably broader for F-actin. In our experiments, however, the major peak occurred at 220 nm, not 232 nm. The difference in the position of this peak compared to the earlier work may be due to the fact that the absorbance does not follow Beer's law below 230 nm (21) at the necessarily high concentrations of ATP and actin (to be above the critical concentration for polymerization) used previously. Improved instrumentation, and the fact that cross-linked actin dimer was studied below its critical concentration in a nonpolymerizing buffer, allowed us to avoid this problem.

Nucleotide Content and Nucleotide Exchange of Cross-linked Actin Dimer—The purified cross-linked actin dimer contained 2 mol of bound nucleotide/mol (Table I). The F-actin from which the cross-linked dimer was prepared contained bound ADP. Since the dimer was isolated and stored in the presence of excess ATP, it was necessary, however, to

![Fig. 2. Sephadex G-200 column chromatography of the de-polymerized cross-linked actin.](image)

![Fig. 3. UV difference spectra of cross-linked actin dimer and native F-actin versus monomeric actin.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Actin analyzed</th>
<th>Concentration</th>
<th>Nucleotide/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>7.6</td>
<td>16.1</td>
</tr>
<tr>
<td>Monomer</td>
<td>19.5</td>
<td>20.2</td>
</tr>
</tbody>
</table>

*The mean ± S.D. for three different preparations of cross-link actin dimer was 2.11 ± 0.04.
determine the nature of the nucleotide bound to the cross-linked actin dimer. This was assessed by determining the rates of exchange of radioactive ATP and ADP into dimer.

After incubation with the radioactive nucleotides for 11 h at 25°C, cross-linked actin dimer had incorporated 1.0 mol of [γ-32P]ATP/mol, 1.1 mol of [α-32P]ATP/mol, and 0.5 mol of [14C]ADP/mol (Fig. 4). The bound [14C]ADP could be rapidly replaced by nonradioactive ATP (Fig. 4). These data indicate that cross-linked actin dimer contains one site per mol that can exchange rapidly with ATP and more slowly with ADP. The incorporation kinetics for this single rapidly exchangeable site can be described by a simple exponential (Fig. 5) with a halftime of 55 min for [γ-32P]ATP and [α-32P]ATP and 5 h for [14C]ADP. The corresponding dissociation rate constants are 1.3 × 10^{-5} min^{-1} and 2.3 × 10^{-5} min^{-1} for ATP and ADP, respectively.

To investigate the ability of the second nucleotide binding site to bind ATP, aliquots of cross-linked actin dimer were incubated for 3 weeks at 4°C with either [γ-32P]ATP or [α-32P]ATP. After removing all unbound nucleotide with Dowex 1, there were 1.92 mol of [γ-32P]ATP/mol of dimer and 1.96 mol of [α-32P]ATP/mol of dimer. Therefore, the second nucleotide binding site can also exchange with ATP, albeit much more slowly than the first site, and both sites, although originally occupied by ADP, will be occupied by ATP when the cross-linked actin dimer is isolated and stored in the presence of excess ATP.

**ADP-P, Content of Cross-linked Actin Dimer**—In the experiments just described, the radioactivity ascribed to bound [γ-32P]ATP could, in fact, have been present as bound ADP-32P, and radioactivity ascribed to bound [α-32P]ATP could have been present as bound [α-32P]ADP-P. To test this possibility for the first nucleotide binding site, three different preparations of cross-linked actin dimer were incubated with [γ-32P]ATP until the rapidly exchangeable site, and only that site, had reached equilibrium, and then unbound radioactive nucleotide was removed by Dowex 1. The total amount of bound radioactive nucleotide corresponded to 1 mol of nucleotide/mol of cross-linked actin dimer (Table II), as expected if radioactive nucleotide had exchanged only with the rapidly exchangeable site. When the dimer-bound radioactivity was further fractionated, 10 to 15% was found to be present as 32P (Table II).

A similar experiment was then carried out in which cross-linked actin dimer was equilibrated with [α-32P]ATP also to the extent of 1 mol/mol. The dimer-bound nucleotides were extracted in 2.5% perchloric acid and separated by thin layer chromatography and the radioactivity in ATP and ADP was determined. The nucleotide fraction was 87% ATP and 11% ADP, in excellent agreement with the previous data for ATP and P. The same results were obtained with cross-linked actin dimer below its critical concentration in buffer G containing 0.5 mM MgCl₂.

In control experiments, when native monomeric G-actin was equilibrated with [γ-32P]ATP and [α-32P]ATP, no more

---

**FIG. 4.** Exchange of the cross-linked actin dimer-bound nucleotide with ATP and ADP. Cross-linked actin dimer (0.8 mg/ml), freed of unbound nucleotide by treatment with Dowex 1, as described in the legend of Table I, was incubated at 25°C in 5 mM Tris/chloride, pH 7.6, and 0.1 mM CaCl₂ with either 0.2 mM [γ-32P]ATP (O), 0.2 mM [α-32P]ATP (C), or [14C]ADP (Δ). Aliquots of 100 μl were removed at the indicated times and added to 40 μl of 30% TCA by the procedure of Lowry et al. (18). After 11 h of incubation (arrow), free radioactive nucleotides were removed from the sample incubated with [14C]ADP by treatment with Dowex 1, 0.2 mM ATP was added, and the loss of actin dimer-bound radioactivity was followed in the same way.

**FIG. 5.** Semilogarithmic plots of the exchange of the cross-linked actin-bound nucleotide with ATP and ADP. The experiment was identical with that described in Fig. 4 except that aliquots were removed more frequently and at earlier times; [γ-32P]ATP (O), [α-32P]ATP (C), [14C]ADP (Δ). Nₛ(t) = moles of nucleotide bound to the rapidly exchangeable site/mol of dimer at time = t; Nₛ(∞) = moles of nucleotide bound to the rapidly exchangeable site/mol of dimer at time = ∞.

**TABLE II**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>(ATP + P₄)/dimer</th>
<th>P₄/dimer</th>
<th>P₄/(ATP + P₄)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol/mol</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>0.98</td>
<td>0.13</td>
<td>13.3</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
<td>0.10</td>
<td>9.8</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>0.14</td>
<td>14.6</td>
</tr>
</tbody>
</table>

Three different preparations of cross-linked actin dimer were incubated in buffer G (1 mg/ml) containing 0.2 mM [γ-32P]ATP of known specific activity at 4°C overnight and then at 25°C for 3 h. The solutions were treated with Dowex 1 to remove unbound nucleotide and the protein concentration and the total bound radioactivity were measured as described in Table I to determine the total moles of bound (ATP + P₄)/mol of dimer. Cross-linked actin dimer-bound P₄ was determined by diluting a 50-μl aliquot into 0.5 ml of water and selectively extracting the P₄ as the phosphomolybdate complex (19).
than 1% or 2% of the total bound radioactivity could be accounted for by \(^{32}\text{P}\)-Pi or \([\alpha-^{32}\text{P}]\text{ADP}\), respectively. These results, therefore, indicate that when cross-linked actin dimer is equilibrated with ATP, about 10 to 15% of the nucleotide bound to the rapidly exchangeable site is present as ADP-P\(_i\).

To determine whether the slowly exchangeable nucleotide binding site also contained bound ADP-\(P\)\(_i\), cross-linked actin dimer containing 1.92 mol of radioactive nucleotide derived from \([\gamma-^{32}\text{P}]\text{ATP}\) was freed of unbound radioactive nucleotide by direct analysis by the method described in Table II. Therefore, the slowly exchangeable nucleotide binding site of cross-linked dimer equilibrated with ATP probably contains significantly less bound ADP-\(P\)\(_i\) than does the rapidly exchangeable nucleotide binding site.

**ATPase Activity of Cross-linked Actin Dimer**—Below its critical concentration, cross-linked actin dimer has a very low ATPase activity (less than 0.03 mol/mol of dimer/h), but it is increased about 35-fold by the addition of 0.5 mM MgCl\(_2\) to a value of 1.1 mol/mol of dimer/h (Table III). This is similar to the behavior previously shown for native monomeric G-actin (9), but the Mg\(^{2+}\)-ATPase activity of dimer is about 7 times greater than that of monomer (Table III). Again, as was previously reported for monomeric actin ATPase activity, the ATPase activity of cross-linked actin dimer was stimulated by cytochalasin D, but only about 3-fold (Table III) to a specific activity about half that of cytochalasin D-stimulated monomeric actin (Table III).

The ATPase activity of cross-linked actin dimer, as of monomer actin (9), was directly proportional to the dimer concentration (data not shown) and the ATPase activity of a mixture of G-actin monomer and cross-linked actin dimer was no more than the sum of their separate activities both in the presence and absence of cytochalasin D (Table III). These data provide further evidence that the ATPase activity and cytochalasin D-stimulated ATPase activity of unpolymerized actin are not the result of actin-actin interactions. That cross-linked actin dimer is capable of interacting with native monomeric actin, a necessary control for the previous experiment was demonstrated by the co-polymerization of monomeric Acanthamoeba \([^{35}\text{S}]\text{actin}\) below its critical concentration with cross-linked actin dimer above its critical concentration. Monomeric \([^{35}\text{S}]\text{actin}\) (0.7 \(\mu\)M) was incubated at 25 °C in 0.5 mM MgCl\(_2\) in buffer G alone and with either nonradioactive G-actin (19.6 \(\mu\)M, critical concentration 3.3 \(\mu\)M) or cross-linked actin dimer (9.8 \(\mu\)M, critical concentration 1.7 \(\mu\)M) and the F-actin was sedimented in the ultracentrifuge. In the absence of the nonradioactive actins, none of the \([^{35}\text{S}]\text{actin}\) was sedimented while 70% and 60% of the radioactivity was recovered with the F-actin formed from nonradioactive monomer and dimer, respectively; the theoretical value for complete co-polymerization is 80 to 85%.

**DISCUSSION**

An F-actin filament contains five different actin protomers distinguishable by their unoccupied actin binding sites (1, 23). As depicted in the heptamer in Fig. 6, there is one terminal subunit (I) with unoccupied sites \(a\) and \(c\), one terminal subunit (VII) with unoccupied sites \(b\) and \(d\), one penultimate subunit (II) with an unoccupied site \(a\), one penultimate subunit (VI) with an unoccupied site \(b\), and multiple internal subunits (III to V) with all four sites occupied. If the actin were polymerized in the presence of ATP, the potential arises for each of the actin protomers to contain a different ratio of ATP, ADP-\(P\)\(_i\), and ADP. The two ends of an actin filament are different. One end (Fig. 6) might be regarded as the unoccupied sites \(a\), \(c\), and \(d\) of the two protomers I and II, and the other end as the unoccupied sites \(b\) and \(d\) of the two protomers VII and VI (or just the \(c\) and \(a\) sites of protomers I and II and the \(d\) and \(b\) sites of protomers VII and VI with which the next monomers to be added would interact).

Within the context of this model, two, and only two, covalently cross-linked dimers can be formed from F-actin (Fig. 6). These are illustrated in the heptamer in Fig. 6 as the III-V dimer and the IV-V dimer. The subunits of one possible dimer are associated through sites \(c\) and \(d\) (the cd dimer), while the subunits of the other are associated through sites \(a\) and \(b\) (the ab dimer). Each of the dimers illustrated in Fig. 6 contains elements resembling both of the two different ends of an actin filament. Each cross-linked dimer contains one subunit with unoccupied sites \(a\) and \(c\), analogous to terminal protomer I, and one subunit with unoccupied sites \(b\) and \(d\), analogous to terminal protomer VII in Fig. 6. The cross-linked cd dimer also contains one ac surface and one bd surface, analogous to the surfaces of protomers I and II and protomers

---

**Table III**

**Hydrolysis of ATP by cross-linked actin dimer and actin monomer in the presence and absence of cytochalasin D**

Cross-linked actin dimer and native monomeric G-actin were incubated alone and together, each below its critical concentration, in buffer G containing 0.5 mM MgCl\(_2\) and 0.22 mM \([\gamma-^{32}\text{P}]\text{ATP}\) for 2.5 h at 25 °C. Then each solution was divided into two parts. One part was made 1.64 \(\mu\)M in cytochalasin D by addition of 164 \(\mu\)M cytochalasin D, and the other was adjusted with the same volume (1%) of dimethylsulfoxide, the solvent for the cytochalasin D. Incubation at 25 °C was continued for 4 h during which samples of 50 \(\mu\)l were removed for the determination of \([^{32}\text{P}]\text{Pi}\). The rates of hydrolysis were constant over the time period assayed.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rate of hydrolysis ((\mu)M (P_i/h))</th>
<th>Specific activity ((\mu)M (P_i/\mu)M actin h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer, 1.0 (\mu)M</td>
<td>1.10</td>
<td>2.9</td>
</tr>
<tr>
<td>Monomer, 0.4 (\mu)M</td>
<td>0.05</td>
<td>2.7</td>
</tr>
<tr>
<td>Dimer, 1.0 (\mu)M + monomer, 0.4 (\mu)M</td>
<td>0.92</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Concentrations of actin monomer were calculated with a molecular weight of 42,000 and concentrations of actin dimer were calculated with a molecular weight of 84,000.*

---

**Fig. 6.** Schematic representation of the associations among protomers in F-actin according to the method of Oosawa and Kasai (1). The bars connecting the protomers represent the covalent links in the two possible cross-linked dimers that might be formed.
VII and VI, respectively, at the two different ends of the filament (Fig. 6).

The cross-linking reagent phenylenebismaleimide reacts initially, rapidly, and stoichiometrically with cysteine 373 and then much more slowly with a lysine amino group, or is hydrolyzed by water (10). Because the reagent molecule is only 1.2 to 1.4 nm in length, it may be able to form only one of the two theoretically possible dimers. Of course, a subset of dimers is possible if more than one reactive lysine on the adjacent actin protomer is close enough to cysteine 373 to react with it. Although we have no direct experimental data on the number or kind of structural isomers that might be present, the purified dimer preparation behaves kinetically as a single species and we believe the isolated cross-linked dimer may prove to be a useful model for one or both ends of a native actin filament. The UV difference spectrum implies that the cross-linked actin dimer may be in a conformation very similar to F-actin, even when the dimer may be in a depolymerizing buffer. The two nucleotide binding sites of cross-linked dimer exchange ATP more slowly than does native monomeric G-actin (24, 25), but more rapidly than the bulk of the protomers of native F-actin (26), as might be anticipated for filament ends. Control experiments showed that the exchange of ATP with monomer derivatized by reaction with phenylenebismaleimide was affected by no more than 25%.

Previously, the presence of bound ADP-Pi on monomeric G-actin (9) and bound ATP and ADP-Pi on filament ends (9, 27) was inferred from indirect evidence. We have now shown that the more rapidly exchangeable of the two nucleotide binding sites of the cross-linked actin dimer contains about 85 to 90% bound ATP and 10 to 15% bound ADP-Pi, when equilibrated with ATP, while the other nucleotide binding site contains ATP but much less, if any, ADP-Pi. Speculatively, the ends of native F-actin filaments, in the presence of excess ATP, may also contain only ATP and ADP-Pi, and the differences in nucleotide binding and nucleotide composition between the two nucleotide binding sites on the cross-linked dimer may be among the important features that functionally differentiate between the two ends of a native filament of F-actin and the monomeric actin species that preferentially add to them. In this regard, it is of considerable interest that at least one of the nucleotide sites on the dimer (the more rapidly exchangeable site) binds ATP in preference to ADP. This raises the possibility that the ends of a native F-actin filament will also preferentially bind ATP over ADP.

Moreover, the potential of filament ends to bind ATP and ADP-Pi, and, as illustrated by the properties of the cross-linked actin dimer to hydrolyze ATP, may be a reflection of an important property common to all of the protomers in an actin filament. It is likely from other evidence (9, 28), that internal protomers in F-actin, which are not represented in the isolated cross-linked actin dimer, also can hydrolyze ATP and also can contain bound ATP and ADP-Pi in addition to bound ATP. An actin protomer, whether within the filament or at the filament ends, might be in different conformations and have different biological properties when its nucleotide site is occupied with ATP or with ADP. Therefore, the apparent ability of actin protomers to convert ATP and ADP may have functional significance in addition to the regulation of the critical concentrations and the rates of polymerization at the two ends of the filament.

Acknowledgments—We thank Dr. Stephen L. Brenner for the [35S]actin, Dr. Lois Greene for the myosin subfragment-1, Dr. Rodney Levine for performing the difference spectroscopy, and Dr. Blair Bowers for the electron microscopy.

REFERENCES