Cross-Link Between cys 374 and cys 10 of Actin Abolishes Polymerizability and Allows Study of the Properties of the "F-Actin Monomer"†

Daniela Heintz and Heinz Faulstich*

Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany

Received April 5, 1995; Revised Manuscript Received October 25, 1995⊗

ABSTRACT: Actin cross-linked between cys 374 and cys 10 via a disulfide-containing bridge, c-A, is completely unpolymerizable even in the presence of phalloidin. Upon the addition of dithiothreitol, c-A polymerizes with high yield, indicating that denaturation due to the modification was almost absent. In the present study we show that cross-linked actin is a useful model for studying the properties of monomeric actin under polymerization conditions. Addition of salt, for example, produced fluorescence changes possibly reflecting conformational transitions but did not lead to the development of phalloidin binding capacity. Cross-linking of the two cysteine residues also caused a decrease in the nucleotide exchange rate by a factor of ca. 3, an effect that was fully reversed by the addition of KCl. Cross-linked actin inhibits DNase I to the same extent as G-actin and binds thymosin β4 and profilin as shown by cross-linking studies. Capping capacity for the barbed end of the filament was not observed, although it might have been expected from the fact that both ends of the cross-link are anchored to subdomain 1. Using the 61-FITC derivative of c-A we showed that c-A is able to bind to myosin S1 with a K_D in the μM range. In agreement with this, c-A shows actomyosin ATPase activity with a K_{app} comparable to that of F-actin, but a V_{max} decreased by a factor of ca. 11. The c-A myosin S1 complex provides the hitherto smallest model of actomyosin, which appears promising for crystallization and X-ray analysis.

Polymeric actin is an important structural element of cells and also serves as a part of the force-generating machine in muscle. However, because of its size the polymeric form of actin is not an easy object for structural or functional studies. A recent, successful approach was the analysis of microfilament structure based on X-ray data of the monomer combined with fiber diffraction data of the polymer (Holmes et al., 1990). Several functional studies performed with actin polymers made use of various kinds of in vitro motility assays (Toyoshima et al., 1989).

Chemically modified actin species which lack the ability to polymerize were suggested as models for studying actin under polymerization conditions. Most of the chemical modifications that were reported to delete polymerizability were located in, or close to, the loop in subdomain 2 (Kabsch et al., 1990). Dansylation of the modified tyrosine in position 69, for example, yielded an actin derivative claimed to be unpolymerizable (Chantler & Gratzer, 1984). Similarly, reaction of lysine in position 61 with fluorescein isothiocyanate (FITC) was reported to abolish polymerizability (Burtnick, 1984). However, subsequent studies by Miki et al. (1987) showed that both actin derivatives were able to polymerize in the presence of phalloidin. Another actin derivative that has been claimed to be unpolymerizable is MBS-actin, which is intramolecularly cross-linked via four thiol- and seven amino groups (Bettache et al., 1989). This actin likewise shows polymerization in the presence of phalloidin (Miki & Hozumi, 1991). Obviously, several actin derivatives exist which appear unpolymerizable but in reality form filaments of extremely low stability. On the addition of phalloidin, such filaments become indistinguishable from normal filaments.

Another chemical modification in subdomain 2 was the reaction of tyrosine in position 53 with diazonium tetrazol (Bender et al., 1976). This actin derivative was found to be unpolymerizable even in the presence of phalloidin; however, it was uncertain whether this actin after the modification was still in its native state. Without any doubt the most desirable actin derivative for such studies would both be non-polymerizable and offer the possibility of cleaving the introduced residue under mild conditions in order to assay the recovered actin for its biological activity. Such a procedure would definitely exclude denaturation as the real cause of non-polymerizability observed for an actin derivative.

To our knowledge, the first actin derivative that meets these conditions is an actin cross-linked between cysteine 10 and cysteine 374 via a disulfide-containing moiety, c-A. This actin species proved to be a useful tool for studying the properties of actin monomers under polymerization conditions. As shown by experiments with myosin S1, c-A can likewise be used for investigating actin-binding proteins under conditions that normally would induce polymerization. Defined complexes of c-A with proteins that normally bind to actin polymers only may be subjected to crystallographic study, a venture that appears hopeless with F-actin.

MATERIALS AND METHODS

Preparation of the Proteins. Actin was prepared according to Pardee and Spudich (1982) with an additional gel filtration step on a TSK HW55 column (Merck) equilibrated with buffer G (2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.02% NaN_3, pH 7.8).

† This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1995.

0006-2960/96/0435-258$12.00/0

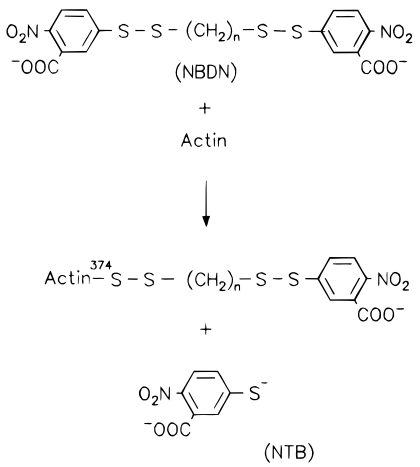

FIGURE 1: Reaction of NBDN with cys 374 in G-actin. The reaction was followed by spectrophotometry of NTB at 412 nm.

Preparation of c-A. G-actin (1.8 mg/mL) in buffer G was reacted with a 10-fold excess of NBDN (Figure 1) for 1 h at 4 °C, and excess reagent was removed by gel filtration on a Bio-Rad P2 column with buffer G. The actin derivative was polymerized at room temperature by the addition of 0.2 mM EGTA/1 mM $MgCl_2$. Removal of ATP was achieved by subsequent incubation with hexokinase (5 units/mL of actin solution) and 0.4 mM glucose for 90 min (Pollard, 1986). After ultracentrifugation at 100 000*g*, the pellets were softened in ADP buffer (2 mM Tris, 1 mM ADP, 0.02% NaN_3, pH 7.8) overnight and, after careful homogenization with a Teflon potter, were stored for an additional 48 h at 0 °C. The cross-linking reaction was followed via release of the yellow NTB anion (ϵ_{412} = 14 150 M^{-1} cm^{-1}). Unreacted actin was separated by the addition of 0.1 M KCl and 1 equiv of phalloidin followed by centrifugation at 100 000*g*. The c-A remaining in the supernatant was further purified on a Bio-Rad P2 column (1.5 × 30 cm) equilibrated with buffer G.

Fluorescent Actins. ^{373}NOD-c-A,[1] ^{61}FITC-c-A, and ^{374}pyrenylactin were prepared as described in the following references (Detmers et al., 1981; Burtnick, 1984; Cooper et al., 1983) and subsequently purified on a Bio-Rad P2 column as described above.

Actin Labeling and Digestion. G-actin and c-A were denaturated with 8 M urea at 95 °C for 2 h and then reacted with a 5-fold excess of [^{14}C]NEM over the number of cysteine residues. Urea and excess reagent were removed on a dialysis against 2% formic acid. Afterward the protein was lyophilized, diluted in 70% formic acid, and subsequently degraded under argon using a 100-fold excess of BrCN over the number of methionine residues for 20 h at room temperature. The solution was diluted 10-fold with water and lyophilized.

Separation of the peptides was achieved on a Sephadex G-50 superfine column (250 × 1.5 cm) equilibrated with 20% acetic acid (Elzinga, 1970; Drewes & Faulstich, 1991a). Aliquots of all fractions were counted for radioactivity.

Other Proteins. Myosin S1 was prepared according to Weeds and Taylor (1976). Thymosin β4 was isolated from bovine lungs following the procedure of Hannappel (1986) and was a gift from Prof. W. Voelter, Tübingen. Profilin was isolated from calf spleen according to Kaiser et al. (1989).

ATPase Measurements. The actin-activated ATPase activity of myosin S1 (0.53 μM) was measured in the presence of varying concentrations of actin or c-A (0−20 μM) in 10 mM imidazole, pH 7.0, 0.5 mM ATP, 15 mM KCL, 0.1 mM $MgCl_2$ at 15 °C. For the measurements under F-actin conditions additional 50 mM KCl was added. These conditions used were described as appropriate to compare the activation of myosin S1 ATPase by G- and F-actin (Offer et al., 1972). The reaction was started by the addition of myosin S1, and during a 10 min incubation aliquots were taken every 2 min. To measure the activation due to G-actin, incubation was extended to 1 h and aliquots were taken every 15 min. The rate of ATP hydrolysis was determined as described by Drewes and Faulstich (1990) using [γ-^{32}P]ATP.

Steady-state ATPase activity of the actin derivatives with F-actin as a control as well as monomer ATPase activity of c-A in comparison to G-actin was determined following the procedures of Stournaras et al. (1990) and Tobacman and Korn (1982), respectively.

Nucleotide Exchange. Nucleotide exchange was measured using 1,N^6-ethenoadenosine 5′-triphosphate (ϵ-ATP) in a buffer containing 2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ϵ-ATP, pH 7.8. Concentration of actin was always 0.3 μM. For the corresponding experiments in the presence of salt, the same buffer containing 0.1 M of KCl or 1 mM of $CaCl_2$ or $MgCl_2$ was used. The reaction was started by the addition of actin to the ϵ-ATP buffer in a Spex Fluorolog (Spex Industries, NY) at room temperature (excitation, 350 nm; emission, 410 nm).

Critical Concentration. Critical concentrations of the different actin derivatives were assayed by subjecting 0.2 mL samples of F-actin in concentrations from 2 to 20 μM to ultracentrifugation at 155 000*g* in a Beckman 42.2 TI rotor for 1 h. For this, actin was polymerized in buffer G by addition of 1 mM $MgCl_2$ for 1 h at room temperature. Protein determination in the supernatant was performed according to Bradford (1976).

Polymerization Measurements. Polymerization of actin was measured in an Ostwald viscometer with an out-flow time of t_0 = 31 s for buffer G at room temperature. Polymerization was started by the addition of 2 mM $MgCl_2$ and 80 mM KCl directly into the viscometer. Further polymerization measurements with c-A (1.55×10^{-4} M) were done in the presence of 2 mM $MgCl_2$ and an equimolar amount of myosin S1 with or without 1.55×10^{-4} M phalloidin. Changes in viscosity were recorded for 12 h.

For monitoring the polymerization of actin in the presence of 30% c-A the fluorescence enhancement of pyrene-labeled actin was used. For this purpose different actin samples doped with 5% pyrenyl−actin were prepared: G-actin (3.4×10^{-5} M); G-actin (3.4×10^{-5} M) + c-A (1.02×10^{-5} M); G-actin (2.38×10^{-5} M) + c-A (1.02×10^{-5} M) and G-actin (4.42×10^{-5} M). Polymerization was started at room temperature by the addition of 1 mM $MgCl_2$ in a Spex Fluorolog spectrofluorimeter (excitation, 366 nm; emission, 406 nm).

[1] Abbreviations: DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; FITC, fluoresceinyl isothiocyanate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimidyl-; NBDN, *n*-nonylene-1,9-bis(5-dithio-2-nitrobenzoic acid); NHS, *N*-hydroxysuccinimide; NOD, 4-nitrobenzo-2-oxa-1,3-diazole; NTB, 2-nitro-5-thiobenzoate.

In further polymerization experiments 30% ^{373}NOD-c-A was added to G-actin to give a 15 μM solution of actin in buffer G, and the polymerization was started in the spectrofluorimeter by the addition of 100 mM KCl (excitation, 470 nm; emission, 530 nm). The analogous experiment was performed with ^{61}FITC-c-A (excitation, 365 nm; emission, 520 nm).

K_D Determination. The fluorescence decrease of ^{61}FITC-c-A (excitation, 365 nm; emission, 400−540 nm) on binding of myosin S1 was used to determine the dissociation constant of c-A and myosin S1 under rigor conditions (40 mM imidazole, 80 mM KCl, pH 7.8) or in the same buffer in the presence of 0.2 mM MgATP. The concentration of ^{61}FITC-c-A was always 2 μM, myosin S1 (A2) was added in 1 μM steps up to a concentration of 12 μM, and the change in fluorescence was recorded for each myosin concentration after 10 min of incubation at room temperature.

Other Experiments. For studying the influence of salt on the conformation of c-A, the fluorescent ^{373}NOD-c-A derivative was used. To a 10 μM solution of ^{373}NOD-c-A in buffer G at room temperature, $CaCl_2$ or $MgCl_2$ was added in steps up to 1.6 mM, KCl up to 0.16 M, and the changes in fluorescence were recorded after 15 min (excitation, 470 nm; emission, 510−560 nm).

DNase I binding to c-A was assayed by using the spectrophotometric procedure and the conditions described by Blikstadt et al. (1978) with G-actin as control for the inhibition of DNase I activity.

Native PAGE was performed as described by Safer et al. (1991), using a buffer containing 25 mM Tris, 0.194 M glycine, 0.3 mM MgATP, 0.1 mM $CaCl_2$.

SDS−PAGE was performed according to Laemmli (1970) using 10% and 12.5% polyacrylamide gels.

Phalloidin binding to c-A was determined using [^{3}H]-demethylphalloin (Puchinger & Wieland, 1969) in an equilibrium dialysis with a Dianorm equipment in the presence of 100 mM KCl for 16 h at 8 °C. c-A was always 6×10^{-6} M, and the toxin was added up to an 100-fold excess over the protein.

The reagent used for the intramolecular cross-link, NBDN, was prepared as described earlier (Faulstich et al., 1992). The 1:1 complex of actin and reagent was confirmed by reduction of the actin derivative with DTT and subsequent determination of the actin concentration ($\epsilon_{290} = 26\,460$ M^{-1} cm^{-1}) and of the concentration of the yellow NTB anion ($\epsilon_{412} = 14\,150$ M^{-1} cm^{-1}), which has no absorption at 290 nm.

RESULTS AND DISCUSSION

Preparation and Purification. The single exposed thiol group of G-actin, cysteine 374, was reacted with *n*-nonylene-1,9-bis(5-dithio-2-nitrobenzoate), NBDN (Figure 1), a bifunctional, thiol-specific reagent recently prepared in our laboratory (Faulstich et al., 1992). Using a 10-fold excess of the reagent at pH 7.8, the reaction between cysteine 374 and one end of the reagent was complete after 1 h as followed from the release of 1 equiv of 2-nitro-5-thiobenzoate, NTB (Figure 1). After separation of excess reagent by gel filtration, the actin conjugate was identified spectrophotometrically as the 1:1 adduct of actin and reagent (see Materials and Methods). The conjugated actin was found to be polymerizable, with a critical concentration of 2.7 μM (control actin: 1.2 μM). In the presence of phalloidin, the critical concentration was 1.6 μM (0.7 μM).

In Ca^{2+}-free buffers containing ADP instead of ATP, actin undergoes a slow conformational change that uncovers cysteine in position 10 (Drewes & Faulstich, 1991a). The corresponding conformational transition also takes place in the actin conjugate. With the increasing exposure of cysteine 10, an intramolecular disulfide bridge is formed yielding actin cross-linked between cys 374 and cys 10 via the nonylene-bis(disulfide) spacer. The cross-linked actin, c-A, was obtained with a yield of up to 82% as shown from the amount of NTB released during the cross-linking reaction. As proven by SDS−PAGE, the reaction results in the formation of monomeric c-A only, without any additional actin dimers or oligomers. The fact that c-A is unpolymerizable even in the presence of phalloidin (see below) was used for the separation of c-A from uncross-linked actin by polymerization of the latter in the presence of KCl and phalloidin. After the purification, preparations of c-A contained ≤1.5% of uncross-linked actin, corresponding to the critical concentration of 1.6 μM for the precursor derivative (see above).

Formation of c-A proceeds only if the bound nucleotide is ADP. In order to improve stability we exchanged, after the cross-linking reaction, bound ADP for ATP. Using the reduction and polymerization assay as described in the following, we found that even after 18 days of storage at 4 °C the ATP complex of c-A showed, after reduction with DTT, no loss of polymerizability.

As known from previous work the uncovering of cysteine 10 observed in Ca^{2+}-free ADP buffer is highly specific, as shown from reaction with 2,4-dinitrophenyl [^{14}C]cysteinyl disulfide, which results in complete labeling of cysteine 10 and cysteine 374 with only minute amounts of labeled cysteine 257 (Drewes & Faulstich, 1991b). Although selective uncovering of cysteine 10 under the given conditions has been shown for several reactions, we assured that the cross-linking reaction investigated in this work had occurred exclusively between cysteine 374 and cysteine 10.

For this, denaturated c-A and G-actin were labeled with [^{14}C]NEM, and the fragments of their cyanogen bromide digests were analyzed by gel chromatography. In agreement with previous studies, four cysteine residues (374, 10, 257, 217) were labeled in normal G-actin, while the fifth, cysteine 285, remained almost inaccessible (5%−10% labeling, left peak of Figure 2A). In the corresponding experiment with c-A (Figure 2B) two of the peaks, attributed by Elzinga (1970) to the fragments containing cysteine 374 and cysteine 10, had disappeared because their SH were no longer available for radioactive labeling due to the disulfide formation while all others remained unchanged. This result shows unambiguously that the cross-link is placed between cysteine 374 and cysteine 10. Additional proof for this finding was obtained by submitting the cyanogen bromide fragments to laser desorption mass spectrometry. In the c-A digest a mass peak at 7260 Da was found, which was absent for the corresponding digest of normal actin and which disappeared in the presence of 2-mercaptoethanol (Figure 2C). This mass value fits well to the cyanogen bromide fragments containing the cross-linked amino and carboxy termini of actin (CB 13 and CB 9), which together have a theoretical mass of 7161 Da.

Additional evidence for the location of the cross-link between cysteine 374 and cysteine 10 came from structural

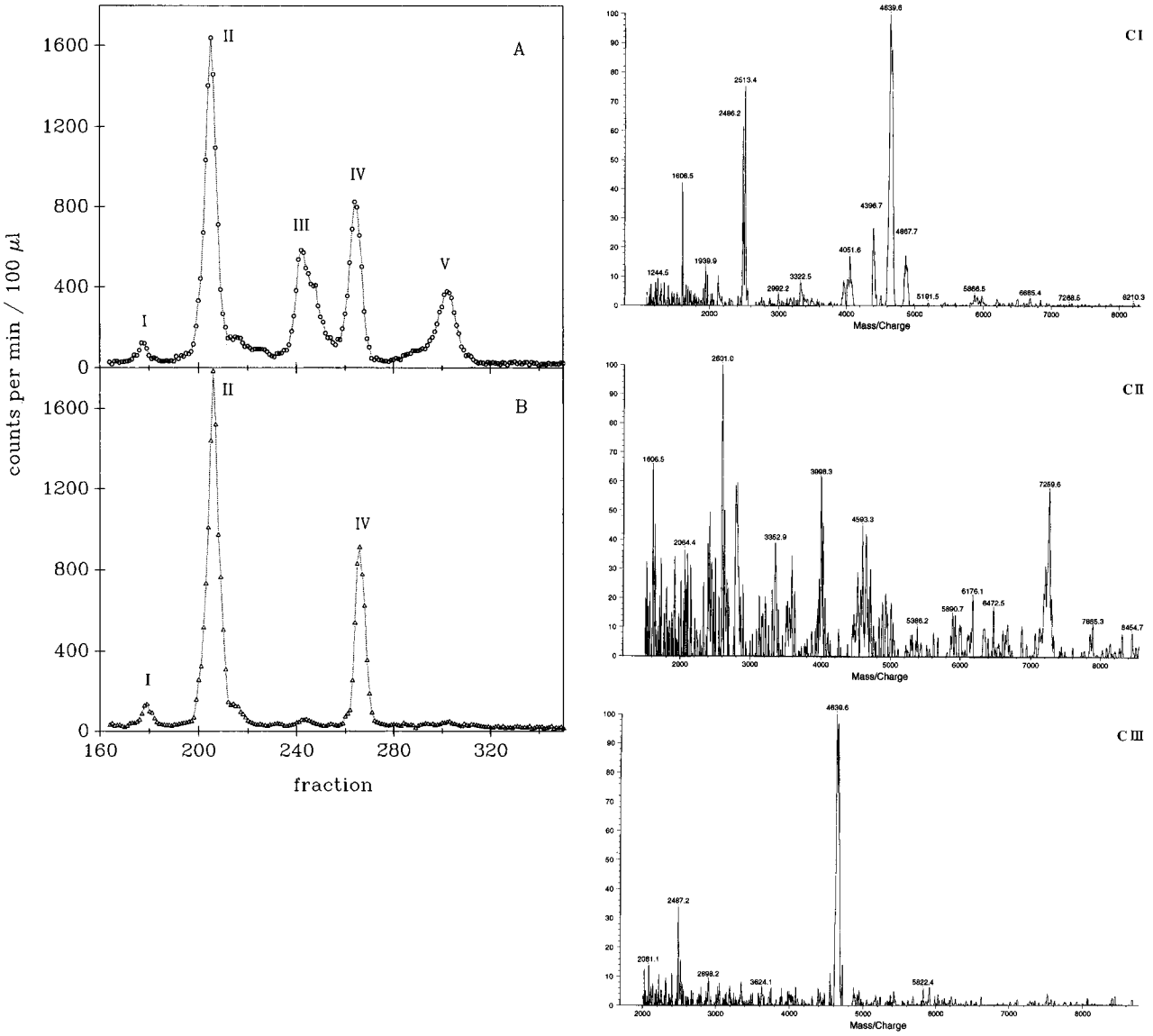

FIGURE 2: Separation of the cyanogen bromide peptides on a Sephadex G-50 SF column (250 × 1.5 cm) of G-actin (A) and c-A (B), denaturated in 8 M urea and labeled with a 5-fold excess of [^{14}C]NEM over the number of SH groups prior to digestion. The labeled fragments contain (from the left) cysteine 285, cysteine 217, cysteine 10, cysteine 257, and cysteine 374. (C): Analysis of the cyanogen bromide peptides with laser desorption mass spectrometry on a Kratos Compact MALDI with a matrix of α-cyanohydroxycinnamic acid: (I) G-actin digest; (II) c-A digest; and (III) c-A digest with 2-mercaptoethanol added.

data of the actin model (Kabsch et al., 1990). According to this, the distance between C_β-372, which is the last C-atom at the C-terminus identified by X-ray analysis, and S_γ of cysteine 10, is 19.5 Å and thus within the effective radius of the cross-linking moiety (18.4 Å). In contrast to this, S_γ of cysteine 257, which is the third cysteine residue that becomes reactive during the unfolding process of nucleotide-free actin (Drewes & Faulstich, 1991b), is at a distance of 38.2 Å, i.e., beyond the reach of the cross-linker. By the same reasons, the reaction with cysteine 217 can be excluded, whose S_γ is located at a distance of 40.1 Å. The only cysteine residue, which, besides cysteine 10, is located within the effective radius of the cross-linker is cysteine 285, whose S_γ is 20.4 Å from C_β-372. This cysteine, however, is almost unreactive in native actin and is only partly accessible even after treatment with 8 M guanidinium hydrochloride. In agreement with this, the rate of radioactive labeling of cysteine 285 was very low and resulted in only a tiny peak (Figure 2), which remained unchanged by the cross-linking reaction.

Polymerizability and Capping Properties. The intramolecular cross-linked actin proved unpolymerizable in the presence of $MgCl_2$ and/or KCl as detected by viscometry and fluorescence measurements. There were no signs of polymerization, even at high protein concentrations (18 mg/mL) or in the presence of myosin S1. Different from other actin derivatives c-A did also not polymerize in the presence of phalloidin. In order to exclude a very slow polymerization of c-A, the viscosity and fluorescence measurements were extended for 12 h. The results show that neither with $MgCl_2$/KCl nor with $MgCl_2$/myosin S1, with or without added phalloidin, was any significant change in viscosity detectable during this time (Figure 3). A similar result was obtained when the polymerizability of ^{373}NOD-c-A was assayed by fluorimetry (Detmers et al., 1981) in an analogous series of experiments (data not shown).

In chemically modified actins unpolymerizability may also be caused by denaturation. We therefore removed the cross-link by reduction with 2-mercaptoethanol and determined the yield of polymerizable actin. In all preparations of c-A

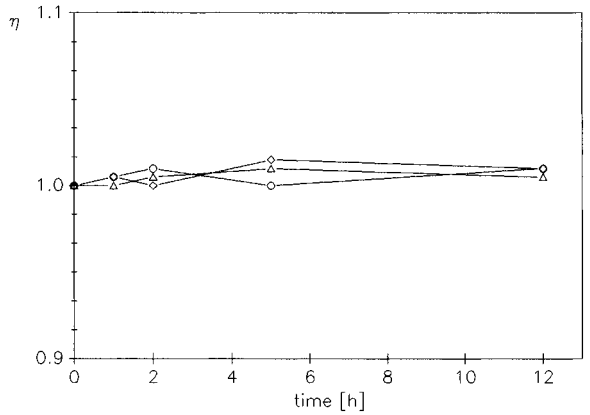

FIGURE 3: Polymerization as followed by viscometry of c-A (1.55 × 10^{-4} M) for 12 h at room temperature (○) in the presence of 2 mM $MgCl_2$ and 80 mM KCl, (◇) in the presence of 2 mM $MgCl_2$ and 1.55 × 10^{-4} M myosin S1, and (△) in the presence of 2 mM $MgCl_2$ plus myosin S1 plus phalloidin, 1.55 × 10^{-4} M each.

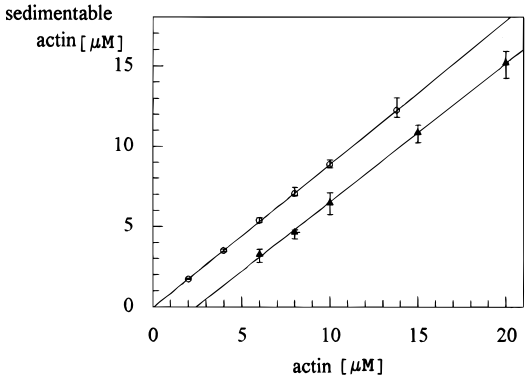

FIGURE 4: Determination of the critical concentration of c-A in the presence of 1 mM $MgCl_2$ immediately after reduction of the disulfide bridge (▲) and after one repolymerization step (○).

treated in this way, yield of F-actin was ca. 85%, indicating that the reactions necessary for introducing and removing the cross-linking moiety had not produced denatured actin in significant amounts. Immediately after reduction of the disulfide moieties the critical concentration was determined to be 2.5 μM. After one repolymerization step the critical concentration was decreased to 0.3 μM, a value that is comparable to that of control actin (Figure 4). The actin obtained after removal of the cross-link was further characterized by measuring the steady state ATPase activity of the polymer. Immediately after reduction, the ATPase activity was 0.51 mol of P_i (mol of actin)$^{-1}$ h^{-1}, but was also decreased to the value of control actin [0.26 mol of P_i (mol of actin)$^{-1}$ h^{-1}] after one repolymerization step. Thus it was proven by two independent methods that neither introduction nor removal of the crosslinking moiety had caused any permanent alteration in the actin molecule.

As reported by Miki and co-workers (1987), chemically modified actin species may appear unpolymerizable but polymerize on the addition of phalloidin. In these cases, the modified actins probably form filaments of very low stability, so that polymerization can be detected only when the filaments are stabilized by phalloidin. Since filament instability is associated with a high steady-state ATPase activity (Drewes & Faulstich, 1990), the ATPase activity of c-A was compared to that of normal F-actin [0.0018 mol of P_i (mol of actin)$^{-1}$ min^{-1}] and was found to be below the limit of detection. For comparison, MBS-actin (Bettache et

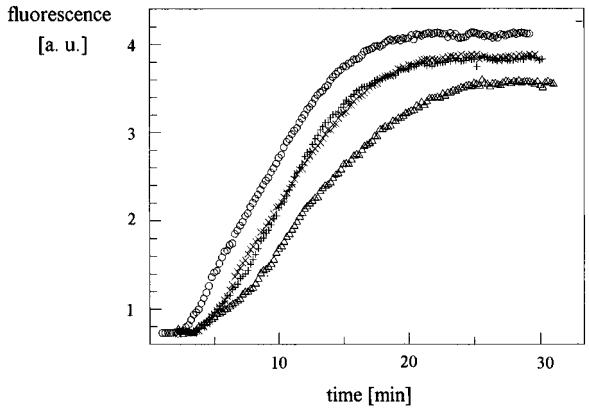

FIGURE 5: Polymerization of actin in the presence of 30% c-A as monitored by the fluorescence enhancement of pyrenyl-actin. Polymerization was started at room temperature in the fluorimeter by the addition of 1 mM $MgCl_2$. (+) G-actin (3.4 × 10^{-5} M); (×) G-actin (3.4 × 10^{-5} M) + c-A (1.02 × 10^{-5} M); (△) G-actin (2.38 × 10^{-5} M) + c-A (1.02 × 10^{-5} M); (○) G-actin (4.42 × 10^{-5} M).

al., 1989), which polymerizes at concentrations ≥1.6 mg/mL or on the addition of phalloidin, was prepared and found to have a steady-state ATPase activity of 0.024 mol of P_i (mol of actin)$^{-1}$ min^{-1}. In order to assay whether c-A possesses ATPase activity at all, we determined its ATPase activity under monomer conditions (Tobacman & Korn, 1982). The ATPase activity of c-A [0.000 95 mol of P_i (mol of actin)$^{-1}$ h^{-1}] was found to be three times lower than that of G-actin (0.0031 mol of P_i (mol of actin)$^{-1}$ h^{-1}) and remained unchanged by the addition of salt.

In c-A both ends of the cross-link are anchored to residues located in subdomain 1. Therefore one would expect that actin−actin contact sites located in subdomains other than 1, for example, in subdomains 2 and 4, remain largely unaffected by the cross-link. If so, c-A might act as a capping protein on the barbed end of the filament. We investigated the polymerization kinetics of pyrenyl-doped actin (5%) in the presence and absence of 30% added c-A. Provided that c-A interferes by blocking the filament growth at the barbed end, kinetics and yield of polymerization should change significantly. In these experiments c-A was found not to influence either rate or degree of polymerization. When 30% of the G-actin was replaced by c-A, the polymerization behavior corresponded to controls in which the actin concentration was reduced to 70% (Figure 5). Also this experiment indicates that c-A does not take part in the polymerization process. In another series of experiments we added ^{61}FITC- or ^{373}NOD-c-A to normal actin in amounts accounting for 30% of the total actin and looked for possible changes in fluorescence after polymerization had been started (data not shown). Again, no changes were seen, not even in the presence of phalloidin, providing additional evidence that both kinds of fluorescent labeled c-A did not take part in the polymerization process. In conclusion, there are no signs that c-A exhibits any capping properties.

Nucleotide Exchange. It is assumed that nucleotide exchange requires a certain degree of flexibility in the actin monomer (Kabsch & Vandekerckhove, 1992). We therefore examined whether the cross-link in subdomain 1 of c-A had any influence on the nucleotide exchange rate. Under G conditions, the nucleotide exchange rate of ATP for ε-ATP was found to be three times lower in c-A than in normal G-actin (Figure 6A), and remained so also under polymer-

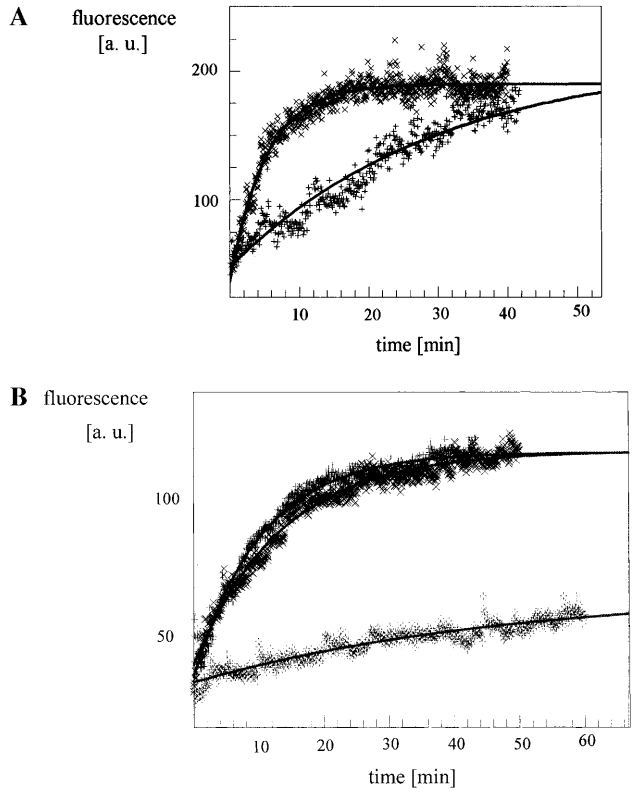

FIGURE 6: (A) Time course of the exchange of ATP bound to G-actin (×) or c-A (+) for ϵ-ATP. The reaction was started by the addition of actin (always 0.3 μM) to the ϵ-ATP buffer (0.2 mM ϵ-ATP, 0.1 mM $CaCl_2$, 0.02% NaN_3, 2 mM Tris, pH 7.8). (B) Effect of KCl on the exchange of ATP bound to c-A for ϵ-ATP. Conditions were the same as in A except that in one experiment the ϵ-ATP buffer contained 100 mM KCl: (*) c-A in the absence of KCl; (×) c-A in the presence of 100 mM KCl; and (+) control G-actin.

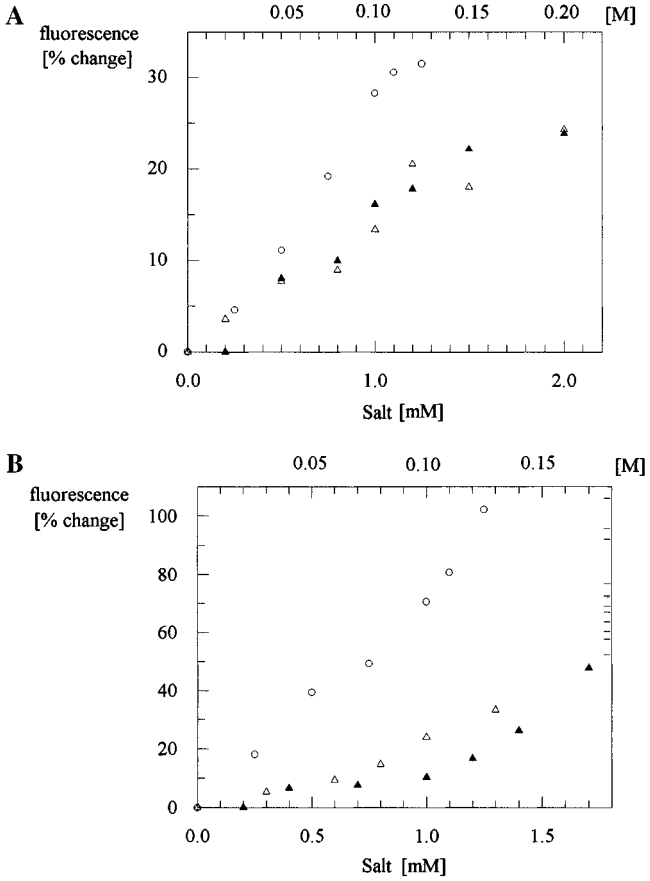

FIGURE 7: Changes in fluorescence (in % change of the base value) as induced by the addition of salt to ^{373}NOD-c-A (10 μM) with Ca^{2+} (A) or Mg^{2+} (B) as the tightly bound ion. $CaCl_2$ (△) or $MgCl_2$ (▲) was added up to 2 mM, and KCl (○) was added up to 120 mM.

ization conditions, for example, after the addition of 1 mM $MgCl_2$ or $CaCl_2$. In contrast, the addition of 0.1 M KCl brought the nucleotide exchange rate back to that of G-actin (Figure 6B). A corresponding series of experiments was performed with actin having Mg^{2+} as the tightly bound ion. Here, the addition of KCl did not accelerate the nucleotide exchange. The differences seen in the effects of K^+ and Mg^{2+}/Ca^{2+} on nucleotide exchange offers c-A as a model system for studying the influence of monovalent and bivalent cations on actin, independent from polymerization. Of interest for further studies may also be the different responses on the addition of KCl depending on whether Ca^{2+} or Mg^{2+} is the tightly bound ion in c-A.

Conformational Changes. Although lysine 373 is located in close vicinity to one of the anchoring points of the cross-link, it was possible to attach the NOD residue (Detmers et al., 1981). The fluorescent reporter group in this position proved useful for studying possible conformational changes induced in c-A on the addition of different salts. In particular, we studied the changes in fluorescence that occur on the addition of $CaCl_2$ or $MgCl_2$ (up to 2 mM) or KCl (up to 120 mM) in c-A containing either Ca^{2+} or Mg^{2+} as the tightly bound cation.

In Ca^{2+}·c-A the addition of bivalent metal ions caused a significant and concentration-dependent increase in fluorescence (Figure 7A) that was virtually independent of whether the bivalent metal ion was Ca^{2+} or Mg^{2+}. The addition of KCl caused a similar but stronger increase in fluorescence, which seemed to plateau at a concentration of ca. 0.1 M KCl.

When c-A contained Mg^{2+} as the tightly bound ion, changes in fluorescence seen on the addition of bivalent metal ions were different and much more pronounced (Figure 7B). Again, KCl had the strongest effect, comparable to that seen in c-A·Ca^{2+}. Similar to the nucleotide exchange experiments, fluorescence measurements using NOD-c-A offer the possibility for studying the effects of monovalent and bivalent cations on monomeric actin independent from polymerization. A more detailed investigation of the salt effects was beyond the scope of this study; further investigations are in progress.

Actin-Binding Proteins, Phalloidin. Since the cross-linking of cysteine 374 and cysteine 10 may have caused conformational changes also in subdomains different from 1, the binding proteins of some actin-binding properties were investigated. The affinity of DNase I, which binds to subdomains 2 and 4, was determined by measuring the inhibition of the enzymatic activity of DNAse I in the presence of c-A. As shown in Figure 8, there is almost no difference between the inhibition caused by c-A and by G-actin. A significant decrease in the inhibitory activity was found, however, when in c-A the bound nucleotide was ADP instead of ATP. This finding is in line with the observation that ADP-G-actin has a decreased affinity to DNase I, which is due to a slightly changed conformation in the ADP-containing protein (Polzar et al., 1989). Obviously, the conformational change which in ADP·G-actin reduces the affinity to DNase I occurs in ADP·c-A as well.

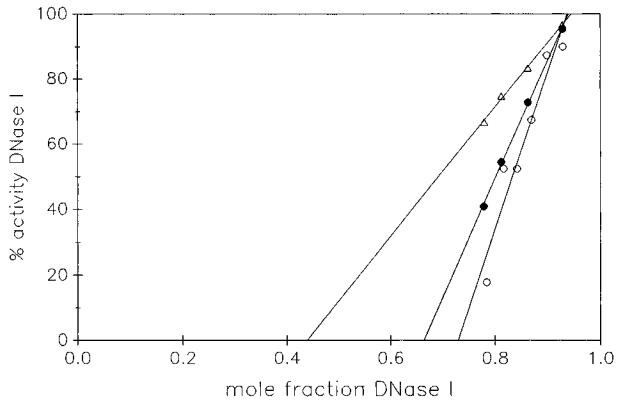

FIGURE 8: Inhibition of DNase I activity, measured at 260 nm according to Blikstad et al. (1978), by G-actin (○), c-A with bound ATP (●), and c-A with bound ADP (△).

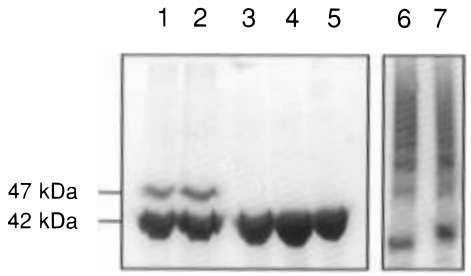

FIGURE 9: Lanes 1−5: SDS−PAGE of c-A cross-linked to Tβ4 (1:1) with EDC/NHS. Lane 1, G-actin and Tβ4 with EDAC/NHS; lane 2, c-A and Tβ4 with EDC/NHS; lane 3, c-A; lane 4, G-actin; lane 5, G-actin with EDC/NHS. Lanes 6 and 7: Native PAGE of c-A in the presence and absence of bovine profilin. Lane 6, c-A and profilin (1:3); lane 7, c-A alone.

Binding of c-A to thymosin β4 was shown by cross-linking experiments. The SDS−PAGE pattern obtained with c-A was indistinguishable from that of normal actin (Figure 9). Binding of profilin to c-A was shown by native gel electrophoresis (Figure 9). Complex formation of both thymosin β4 and profilin with c-A is surprising because both proteins were reported to bind to subdomain 1 (Vandekerckhove et al., 1989; Heintz et al., 1993). Again, the limited frame of this study did not allow investigation of the complexes of thymosin β4 and profilin with c-A in more detail.

The affinity of phalloidin to c-A under polymerization conditions was assayed in an equilibrium dialysis experiment using [^{3}H]demethylphalloin. We found that c-A is unable to bind the toxin even at a 100-fold excess of phalloidin (data not shown). Since one side of the cross-linking moiety is anchored at the C-terminus, it cannot be excluded that the cross-link affects the binding site of the toxin reported to be located in close vicinity to the C-terminus (Vandekerckhove et al., 1985; Faulstich et al., 1993; Lorenz et al., 1993). Alternatively, binding of phalloidin may require the association of two or three actin subunits, which is not possible for c-A.

Binding to Myosin S1. Myosin S1 preferentially binds to F-actin, but likewise has affinity to G-actin and is able to induce polymerization (Miller et al., 1988). The binding site of myosin S1 was identified in subdomain 1 (Rayment et al., 1993). From cross-linking experiments under rigor conditions using EDC/NHS we were able to demonstrate that c-A is still able to bind to myosin S1 (Figure 10). Thus in SDS−PAGE two high molecular weight bands were

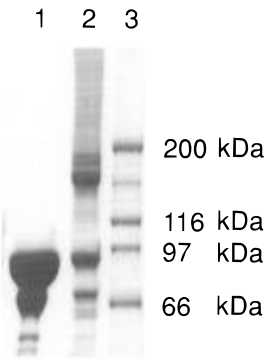

FIGURE 10: SDS−PAGE pattern of the cross-linking reaction of c-A and myosin S1 with EDC/NHS. Lane 1, myosin S1; lane 2, c-A and myosin S1 (1:1) with EDC/NHS; lane 3, molecular weight marker.

Table 1: Actin Activation of Myosin S1 ATPase[a]

	V_{max} [μmol of P_i (μmol of S1)$^{-1}$ min^{-1}]	K_{app} (μM)
F-actin	248 ± 13	5.8 ± 0.2
c-A (F conditions)	23 ± 2.5	6.1 ± 0.3
G-actin	3.8 ± 0.3	11.9 ± 0.3
c-A (G conditions)	3.4 ± 0.4	12.4 ± 0.5

[a] Activation of myosin S1 ATPase by c-A under G and F conditions in comparison to G- and F-actin was assayed according to Offer et al. (1972). The ATPase activity of myosin S1 alone was found to be 1.2 and 0.71 μmol of P_i (μmol of S1)$^{-1}$ min^{-1} under F and G conditions, respectively. The values of the actin-activated myosin S1 ATPase were reduced for these values before the determination of V_{max} and K_{app}.

detected (162 and 180 kDa), exhibiting a pattern that was nearly identical to that obtained by Combeau et al. (1992) in a similar experiment using normal G-actin. According to these authors the two bands represent conjugates of actin and myosin S1. By Western blotting we showed the presence of actin in these bands (no picture). This result is surprising since the main binding site of myosin S1 is located in the same subdomain as the modification.

Decrease of ^{61}FITC-fluorescence caused by complex formation of c-A with myosin S1 was used for measuring the K_D values in the absence and presence of ATP. Under rigor conditions, the K_D was determined as 0.8 ± 0.1 μM, whereas in the presence of ATP the K_D value was 3 ± 0.6 μM. As compared to the corresponding values reported for G-actin, 0.05 μM in the absence (Lheureux et al., 1993) and 0.4 μM in the presence of ATP (Chaussepied & Kasprzak, 1989), binding of c-A to myosin S1 is distinctly weaker. Although the K_D values of the two series of experiments cannot be compared directly because of the great differences in ionic strength, it appears that the cross-link in subdomain 1 causes a decrease in affinity to myosin S1. A comparison with K_D values of F-actin is likewise problematic, since it has been reported that myosin S1 may form contacts with two monomers along the helix (Trayer, 1993).

Although unpolymerizable even in the presence of myosin S1, c-A activates actomyosin ATPase. This is different for MBS- and ^{61}FITC-actin, which both showed no activation of actomyosin ATPase in their monomeric form (Miki, 1989; Miki & Hozumi, 1991). As shown in Table 1, c-A under F conditions has a K_{app} value comparable to that of F-actin, but the V_{max} is decreased by a factor of 11. A comparison of the values obtained for G-actin and c-A under G conditions demonstrate, that there is virtually no difference.

The ATPase and affinity data suggest that the complex of myosin S1 and c-A represents a model of the actomyosin ATPase, probably the smallest known so far, and a promising candidate for crystallization experiments.

ACKNOWLEDGMENT

The authors gratefully acknowledge a gift of thymosin β4 by W. Voelter and M. Mihelic, Tübingen, Germany, and thank W. Kabsch for the distance calculations and S. Stoeva for recording the mass spectra.

REFERENCES

Bender, N., Fasold, H., Kenmoku, A., Middelhoff, G., & Volk, K. E. (1976) *Eur. J. Biochem. 64*, 215−218.
Bettache, N., Bertrand, R., & Kassab, R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6028−2032.
Bettache, N., Bertrand, R., & Kassab, R. (1990) *Biochemistry 29*, 9085−9091.
Blikstad, I., Markey, L., & Carlsson, L. (1978) *Cell 15*, 935−943.
Bradford, M. (1976) *Anal. Biochem. 72*, 248−252.
Burtnick, L. D. (1984) *Biochim. Biophys. Acta 791*, 57−62.
Chantler, P. D., & Gratzer, W. B. (1984) *Eur. J. Biochem. 60*, 67−72.
Chaussepied, P., & Kasprzak, A. A. (1989) *Nature 342*, 950−953.
Combeau, C., Didry, D., & Carlier, M. F. (1992) *J. Biol. Chem. 277*, 14038−14046.
Cooper, J. A., Buhle, E. L., Walker, S. B., Tsong, T. Y., & Pollard, T. D. (1983) *J. Muscle Res. Cell Motil. 4*, 253−262.
Detmers, P., Weber, A., Elzinga, M., & Stephens, R. A. (1981) *J. Biol. Chem. 256*, 99−105.
Drewes, G., & Faulstich, H. (1990) *J. Biol. Chem. 265*, 3017−3021.
Drewes, G., & Faulstich, H. (1991a) *J. Biol. Chem. 266*, 5508−5513.
Drewes, G., & Faulstich, H. (1991b) *Anal. Biochem. 188*, 109−113.
Drewes, G., & Faulstich, H. (1991c) *J. Muscle Res. Cell Motil. 12*, 106−106.
Elzinga, M. (1970) *Biochemistry 9*, 1365−1374.
Faulstich, H., Heintz, D., & Drewes, G. (1992) *FEBS Lett. 302*, 201−205.
Faulstich, H., Zobeley, S., Heintz, D., & Drewes, G. (1993) *FEBS Lett. 318*, 218−222.
Hannappel, E. (1986) *Anal. Biochem. 156*, 390−396.
Heintz, D., Reichert, A., Mihelic, M., Voelter, W., & Faulstich, H. (1993) *FEBS Lett. 329*, 9−12.
Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44−49.
Kabsch, W., & Vandekerckhove, J. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 49−76.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37−44.
Kaiser, D. A., Goldschmidt-Clermont, P. J., Levine, B. A., & Pollard, T. D. (1989) *Cell Motil. Cytoskeleton 14*, 251−262.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lheureux, K., Forne, T., & Chaussepied, P. (1993) *Biochemistry 32*, 10005−10014.
Lorenz, M., Popp, D., & Holmes, K. C. (1993) *J. Mol. Biol. 234*, 826−836.
Miki, M. (1989) *J. Biochem.* (*Tokyo*) *106*, 651−655.
Miki, M., & Hozumi, T. (1991) *Biochemistry 30*, 5625−5630.
Miki, M., Barden, J. A., Dos Remedios, C. G., Phillips, L., & Hambly, B. D. (1987) *Eur. J. Biochem. 165*, 125−130.
Miller, L., Phillips, M., & Reisler, E. (1988) *J. Biol. Chem. 263*, 1996−2002.
Offer, G., Baker, H., & Baker, L. (1972) *J. Mol. Biol. 66*, 435−444.
Pardee, J., & Spudich, J. A. (1982) *Methods Enzymol. 85*, 164−181.
Pollard, T. D. (1986) *J. Cell Biol. 103*, 2747−2754.
Polzar, B., Novak, E., Goody, R. S., & Mannherz, H. G. (1989) *Eur. J. Biochem. 114*, 267−275.
Puchinger, H., & Wieland, T. (1969) *Liebigs Ann. Chem. 725*, 238−240.
Rayment, I., Holden, H. M., Whittaker, M., Yohn, C., Lorenz, M., Holmes, K. C., & Milligan, R. A. (1993) *Science 261*, 58−65.
Safer, D., Elzinga, M., & Nachmias, V. T. (1991) *J. Biol. Chem. 226*, 4029−4032.
Stournaras, C., Drewes, G., Blackholm, H., Merkler, I., & Faulstich, H. (1990) *Biochim. Biophys. Acta 1037*, 86−91.
Trayer, I. P. (1993) *Nature 364*, 101−103.
Tobacman, L. S., & Korn, E. D. (1982) *J. Biol. Chem. 257*, 4166−4170.
Toyoshima, Y. T., Toyoshima, C., & Spudich, J. A. (1989) *Nature 341*, 154−156.
Vandekerckhove, J. A., Deboben, A., Nassal, M., & Wieland, T. (1985) *EMBO J. 4*, 22815−22818.
Vandekerckhove, J., Kaiser, D. A., & Pollard, T. D. (1989) *J. Cell Biol. 109*, 619−626.
Weeds, A. G., & Taylor, R. S. (1976) *Nature 257*, 54−57.

BI950765X