

Proteolytic removal of three C-terminal residues of actin alters the monomer–monomer interactions

Malgorzata MOSSAKOWSKA,* Joanna MORACZEWSKA,* Sofia KHAITLINA† and Hanna STRZELECKA-GOLASZEWSKA*‡

*Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, PL-02-093 Warsaw, Poland,

and †Department of Cell Culture, Institute of Cytology, St. Petersburg, Russia

Homogeneous preparations of actin devoid of the three C-terminal residues were obtained by digestion of G-actin with trypsin after blocking proteolysis at other sites by substitution of Mg^{2+} for the tightly bound Ca^{2+} . Removal of the C-terminal residues resulted in the following: an enhancement of the Mg^{2+} -induced hydrolysis of ATP in low-ionic-strength solutions of actin; an increase in the critical concentration for polymer-

ization; a decrease in the initial rate of polymerization; and an enhancement of the steady-state exchange of subunits in the polymer. Electron microscopy indicated an increased fragility of the filaments assembled from truncated actin. The results suggest that removal of the C-terminal residues increases the rate constants for monomer dissociation from the polymer ends and from the oligomeric species.

INTRODUCTION

The C-terminus of the actin polypeptide chain has been implicated in the intermonomer interactions in the polymer [1], and it has been shown to be close to one of the interfaces for binding of the myosin head and to contact sites for certain actin-binding proteins (for reviews see [2,3]). Therefore, studies on actin which is devoid of the C-terminal residues may provide information on the structure–function relationships in this protein. Investigation of the properties of C-terminal-truncated actin also seems to be important because, in the G-actin–DNAase I crystals used to determine the three-dimensional structure of the actin monomer [4], which, in turn, served to construct the atomic model of the actin filament [1], the three C-terminal residues of the actin polypeptide chain were missing.

To date, C-terminal-truncated actin has been obtained by digestion of G-actin with carboxypeptidase A to remove the C-terminal phenylalanine residue [5,6], or by digestion of F-actin with trypsin [7,8]. This latter enzyme specifically cleaves the peptide bonds between residues 372 and 373 and residues 373 and 374 in the C-terminal segment, as well as those between residues 62 and 63 and residues 68 and 69 [9–11]. In F-actin [9], as in the G-actin–DNAase I complex [12], the bonds between residues 62 and 63 and 68 and 69 are, however, protected from proteolysis. Based on identification of residues released by carboxypeptidase B from trypsin-digested F-actin, it has been reported recently that the polymerization also results in shielding of the bond between residues 372 and 373, and thus the trypsin cleavage of F-actin yields the derivative devoid of the two C-terminal residues [8].

We have observed recently [13] that substitution of Mg^{2+} for Ca^{2+} at the high-affinity site for metal ion binding in G-actin strongly inhibited the tryptic cleavages at Arg-62 and Lys-68, whereas proteolysis of the peptide bonds between Lys-373 and Cys-374 and between Arg-372 and Lys-373 was less influenced. Based on these observations we have developed a simple pro-

cedure for obtaining homogeneous preparations of actin devoid of the three C-terminal amino-acid residues. In this paper we describe this procedure and report on certain properties of the truncated actin.

MATERIALS AND METHODS

Protein preparations

Rabbit skeletal muscle actin was prepared according to Spudich and Watt [14] and was stored in buffer containing 2 mM Hepes, pH 7.6, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol and 0.02% NaN_3 (buffer G). Actin, fluorescently labelled at Cys-374 with *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine (1,5-IAEDANS) was prepared according to [15]. Actin labelled with 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole (NBD) chloride was obtained by the method of Detmers et al. [16]. Preparations of Ca–G-actin (actin in buffer G) were converted into Mg–G-actin by incubation for 10 min with 0.2 mM EGTA/0.1 mM $MgCl_2$ after a 2-fold dilution with Ca^{2+} -free buffer G to lower the $CaCl_2$ concentration to 50 μ M.

Actin devoid of the three C-terminal residues was obtained by digestion of 24–28 μ M Mg–G-actin with trypsin at an enzyme/protein mass ratio of 1:50 for 60 min at 25 °C. The reaction was terminated by adding soybean trypsin inhibitor (SBTI) at twice the trypsin concentration. The truncated actin was polymerized with 0.1 M KCl/2 mM $MgCl_2$, collected by centrifugation at 150000 *g* for 2 h, and depolymerized by homogenization of the pellets in buffer G, followed by overnight dialysis against the same buffer. The G-actin solution was clarified by centrifugation for 45 min at 100000 *g*. The truncated actin migrated on SDS/polyacrylamide gels as a single band with a mobility indistinguishable from that of intact actin.

Light-scattering and fluorescence measurements

Light scattering and fluorescence were measured in a

Abbreviations used: SBTI, soybean trypsin inhibitor; 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; NBD, 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole; 1,4-PBM, *NN'*-1,4-phenylenebismaleimide; ϵ ATP, 1, *N*⁶-etheno-ATP; LD, lower dimer; UD, upper dimer; AEDANS, acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine.

‡ To whom correspondence should be sent.

Perkin-Elmer LS-5b luminescence spectrometer. The fluorescence excitation and emission wavelengths were 340 and 460 nm respectively, for AEDANS-actin, and 470 nm and 530 nm for NBD-labelled actin. Fluorescence intensity of 1,*N*⁶-etheno-ATP (ϵ -ATP) was measured at 410 nm after excitation at 350 nm.

Other methods

Viscosity was measured in Ostwald-type viscometers with an outflow time of 30–40 s for water at 23 °C.

Protein concentration was measured spectrophotometrically at 290 nm using absorption coefficients of 0.63 and 0.66 ml·mg⁻¹·cm⁻¹ for G- and F-actin respectively [17]. The concentration of AEDANS-actin was calculated according to [18], and that of NBD-actin was determined by the method of Bradford [19].

The total number of thiol groups in actin was determined by the reaction with β -hydroxyethyl-2,4-dinitrophenyl disulphide [20] in the presence of 2.5 M guanidine hydrochloride using an absorption coefficient of 12.6×10^3 M⁻¹·cm⁻¹ at 408 nm for the released 2,4-dinitrophenol [21].

Inorganic phosphate was determined by the method of Baginski et al. [22], modified as described in [23].

SDS/PAGE was carried out according to Laemmli [24] on either 10% (w/v) or 15% (w/v) polyacrylamide gel slabs. To visualize fluorescent protein bands the gels were photographed in a u.v.-light transilluminator before staining. Densitometric scans of Coomassie Blue-stained protein bands were performed in a Shimadzu CS-9000 Dual-Wavelength Flying-Spot Scanner.

Samples for electron microscopy were negatively stained with 1% (w/v) uranyl acetate after dilution to a protein concentration of 0.1 mg/ml. Specimens were examined in a Jeol JEM 1200 EX electron microscope at an accelerating voltage of 80 kV.

RESULTS

Preparation of C-terminal-truncated actin

Proteolytic removal of the C-terminal dipeptide Cys-374–Phe-375 from the actin polypeptide chain can be conveniently

Table 1 Comparison of time-courses of the tryptic removal from G-actin of AEDANS-labelled and non-labelled Cys-374

AEDANS-labelled and non-labelled Mg-G-actin (24 μ M) were digested with trypsin at an enzyme/protein mass ratio of 1:50 at 25 °C. Changes in the fluorescence intensity of the AEDANS-labelled actin were recorded as described in the Materials and methods section. The results presented in the Table were calculated taking the initial fluorescence intensity as 100%, and the final plateau value as 0%. In samples of non-labelled actin the reaction was terminated at the times indicated by addition of SBTI at twice the concentration of trypsin. The truncated actin was separated from the amino-acid residues released by trypsin by passing through Sephadex G-25 columns (10 mm \times 70 mm), and thiol groups were determined in the presence of 2.5 M guanidine hydrochloride as described in the Materials and methods section.

Time of digestion (min)	Fluorescence of AEDANS-labelled actin (%)	Total thiol groups in non-labelled actin (mol/mol)
0	100	4.98
1	58	4.62
2	46	4.45
7	22	4.22
11	10	4.00
20	2	4.17

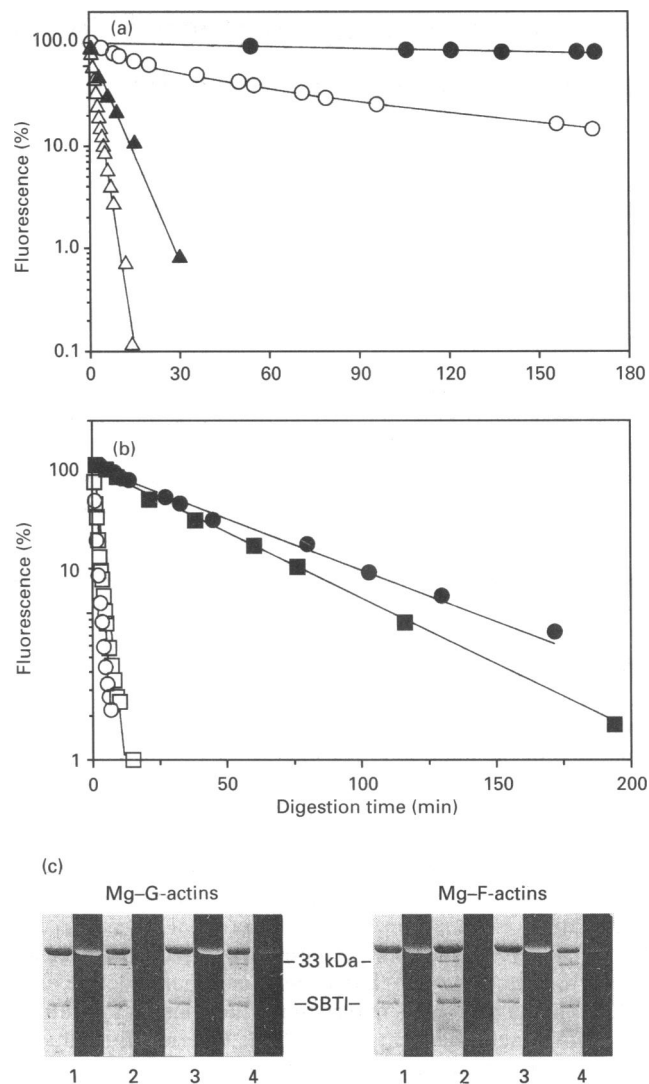


Figure 1 Time-course of changes in the fluorescence intensity of AEDANS-labelled (open symbols) and NBD-labelled (closed symbols) actins during digestion with trypsin

The actins (24 μ M) were digested in the fluorescence cuvettes at 25 °C, at a trypsin/protein mass ratio of 1:50 (a) or 1:5 (b), before and after polymerization with 0.1 M KCl. The fluorescence intensities were normalized to 100% at time zero and 0% when the final plateau value was reached. (Δ , \blacktriangle), Mg-G-actin; (\circ , \bullet), Mg-F-actin; (\square , \blacksquare), Ca-F-actin. (c) SDS/PAGE of AEDANS-labelled (lanes 1 and 2) and NBD-labelled actins (lanes 3 and 4), undigested (lanes 1 and 3) or digested with trypsin until the fluorescence intensity reached the final plateau value (lanes 2 and 4). Coomassie Blue-stained protein bands and fluorescence profiles of the gels are shown.

monitored by using actin labelled with 1,5-IAEDANS at Cys-374 and following the decrease in its fluorescence intensity. Table 1 shows that there is a good correlation between the decrease in the fluorescence intensity of the labelled Mg-G-actin and the decrease in the total number of thiol groups from five to four in non-labelled Mg-G-actin during digestion with trypsin under identical conditions. This observation indicates that labelling with 1,5-IAEDANS does not affect the cleavage rate. To monitor proteolysis of the peptide bond between Arg-372 and Lys-373, we have used actin which has been fluorescently labelled with NBD under conditions in which this probe preferentially reacts with Lys-373 [16]. Comparison of time-courses of the fluorescence decrease during tryptic digestion of AEDANS- and NBD-

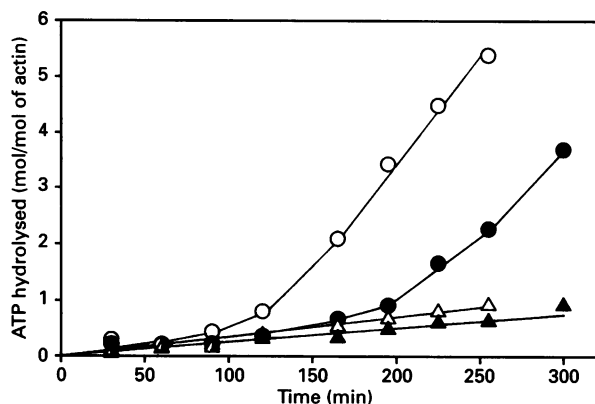


Figure 2 ATP hydrolysis in solutions of truncated and intact Mg-G-actin

Actin solutions (24 μ M) in buffer G containing 50 μ M CaCl₂ were supplemented with 0.2 mM EGTA/0.1 mM MgCl₂ at time zero. The solutions were continuously shaken using a shaking thermostat (open symbols) or left standing without shaking (closed symbols) at 23 °C. At time intervals the concentration of inorganic phosphate was determined. (Δ , \blacktriangle), intact actin; (\circ , \bullet), truncated actin.

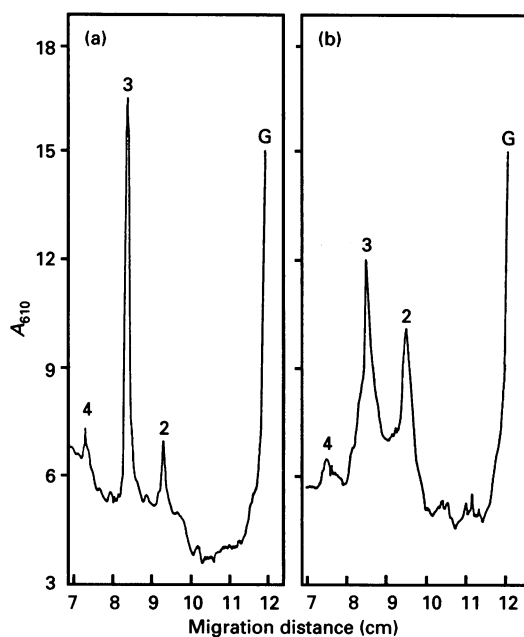


Figure 3 SDS/PAGE patterns of glutaraldehyde-cross-linked actin

Actin solutions (52 μ M) in buffer G containing 50 μ M CaCl₂ were incubated with 0.2 mM EGTA/0.1 mM MgCl₂ for 2 h and then treated with 5 mM glutaraldehyde for 1 h. Cross-linked dimers (2), trimers (3) and tetramers (4) were identified based on their molecular masses calculated from a standard curve obtained with a Pharmacia molecular-mass kit (14–94 kDa proteins). The front of the monomer band is labelled G. (a) Intact actin; (b) truncated actin.

labelled actins (Figure 1) indicates that the Cys-374–Phe-375 dipeptide and Lys-373 are removed sequentially. An alternative explanation for the slower decrease in the fluorescence of NBD-actin compared with AEDANS-actin is that the NBD labelling diminishes the accessibility of the C-terminal segment to trypsin. Whichever of these explanations is true, it is evident from Figure 1 that proteolysis of the bond between Arg-372 and Lys-373 is slowed by polymerization of actin to a much greater extent than is the release of the Cys-374–Phe-375 dipeptide. The

rate of release of NBD-labelled Lys-373 from Mg-G-actin was only 2–3 times lower, whereas in KCl-polymerized actins, irrespective of whether Ca²⁺ or Mg²⁺ was the tightly bound cation, it was about 50-fold lower than the rate of splitting off of residues 374 and 375. Based on these observations, homogeneous preparations of actin devoid of the three-residue C-terminal segment can be obtained by digestion of Mg-G-actin with trypsin as described in the Materials and methods section. The residual fluorescence of actin in the digests of NBD-actin, which were subjected to SDS/PAGE at the fluorescence plateau (Figure 1c, lane 4), undoubtedly resulted from labelling of residues other than Lys-373 [16], as a prolonged digestion produced no further change.

Truncated G-actin, like intact actin, could be stored for several days at 0 °C without any detectable loss of activity if Ca²⁺ was the tightly bound cation. The viscosity of truncated actin polymerized with KCl after storage for 3 days in the Mg-bound form was, however, only about 60% of the value obtained for this actin polymerized immediately after substitution of Mg²⁺ for the bound Ca²⁺, whereas in solutions of intact Mg-G-actin only about 6% of the protein was denatured within this time. This observation points to the importance of conversion of the preparations of truncated Mg-actin into the Ca-bound form immediately after their purification.

Mg²⁺-induced oligomers of truncated actin

Earlier studies have revealed that solutions of actin below the critical concentration for polymerization hydrolyse ATP in the presence of Mg²⁺ [25,26]. As shown in Figure 2, within 1–2 h of substitution of Mg²⁺ for the bound Ca²⁺, ATP hydrolysis in solutions of the truncated and intact actin proceeded with a similar, low rate of 0.1–0.2 mol/h per mol of actin. During longer incubations the liberation of P_i in the solution of intact actin remained linear, whereas its rate in the solution of truncated actin increased with time. The onset of the enhanced ATP hydrolysis was accelerated when the solution was continuously shaken. Similar treatment of intact Mg-G-actin had only a slight effect on the rate of P_i liberation. The enhanced hydrolysis of ATP could result in the conversion of ATP-G-actin into ADP-G-actin, which would explain the relatively fast inactivation of truncated G-actin in its Mg-bound form.

The acceleration of the Mg²⁺-dependent ATP hydrolysis upon removal of the C-terminal residues suggests increased rates of both formation and dissociation of the Mg²⁺-induced oligomers [26–29] because the P_i liberated within a few hours exceeded 1 mol/mol of total actin. As shown in Figure 3, the oligomers formed in concentrated actin solutions during prolonged incubation with MgCl₂/EGTA can be detected by SDS/PAGE after cross-linking with glutaraldehyde [30]. Relative amounts of the glutaraldehyde-fixed oligomers in preparations of truncated and intact actin, estimated by measuring intensities of Coomassie Blue-stained bands, were similar. There was, however, a marked difference in the molecular-mass distribution: in intact actin, oligomers with an apparent molecular mass of about 115 kDa, close to that of a trimer, were the predominant cross-linked species, whereas in preparations of truncated actin, 86 kDa and 115 kDa products of cross-linking were present in an approx. 1:1 ratio. Although the size of the cross-linked species is not a measure of the real size of oligomers present in solution, these results may be taken as an indication that the equilibrium between various oligomeric forms is shifted toward oligomers of smaller size when actin lacks the C-terminal residues. An alternative possibility is that the cross-linking products with apparent molecular masses of 86 kDa and 115 kDa both rep-

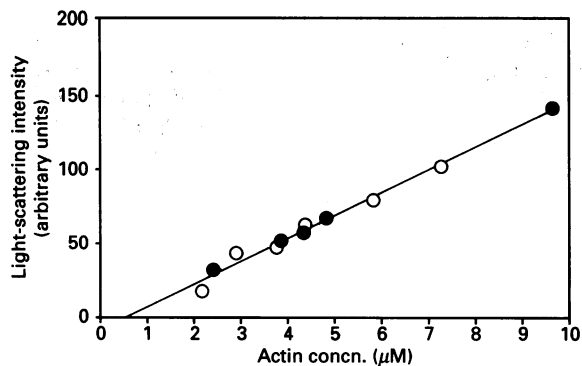


Figure 4 Polymerization of truncated actin as a function of protein concentration

Truncated Ca-G-actin was converted into Mg-G-actin (see the Materials and methods section) and then polymerized with 50 mM KCl at the protein concentrations indicated on the abscissa. The steady-state extent of polymerization at 25 °C was determined by measuring the increase in the light-scattering intensity at 90° at 360 nm. Closed and open symbols denote the results from two independent experiments.

resent dimers which differ in the type of intermonomer contacts. Formation of two types of cross-linked dimers, referred to as 'lower dimer' (LD, 86 kDa) and 'upper dimer' (UD, 115 kDa), has been documented for intact actin under polymerizing conditions, with the use of *NN'*-1,4-phenylenebismaleimide (1,4-PBM) as the cross-linking reagent [31]. These authors have noted that a similar pattern of cross-linking products and a similar shift in this pattern (from LD to UD) with time after addition of polymerizing salt could be observed using glutaraldehyde. In the 1,4-PBM-induced UD, the cross-link is between Cys-374 and Lys-191 [32] on the neighbouring molecules along the F-actin genetic helix [1], whereas the monomer-monomer interfaces in the LD seem to correspond to interfilament contacts in the paracrystalline assemblies [31].

No significant changes in the relative amount and size distribution of the cross-linked oligomers were produced by stirring of the solutions during pre-incubation with MgCl₂/EGTA (results not shown). Thus, whatever is the nature of the intersubunit contacts in the Mg-induced oligomers, the acceleration of ATP hydrolysis upon agitation of the solutions of truncated actin seems to reflect increased dynamics of the oligomers of this actin due to their mechanical instability.

Polymerization of truncated actin

The critical monomer concentration for polymerization of truncated Mg-G-actin in 50 mM KCl, determined by measuring the increase in light scattering as a function of actin concentration (Figure 4), is 0.5 µM. In agreement with published results [33] for intact Mg-G-actin, the value of 0.1 µM was obtained by measuring the increase in pyrenyl fluorescence [34] under similar conditions.

Polymerization kinetics were studied with the light-scattering method and by following an enhancement of the fluorescence of intact NBD-labelled G-actin [16] added at subcritical concentrations. The initial rates of increase in both the NBD fluorescence and light-scattering intensity of the solutions of truncated actin were lower than those for intact actin, although the final values were reached faster by truncated actin. These differences were best seen when the polymerization was slowed by using low concentrations of KCl (Figure 5a). Only at high concentrations of protein were the initial rates of polymerization

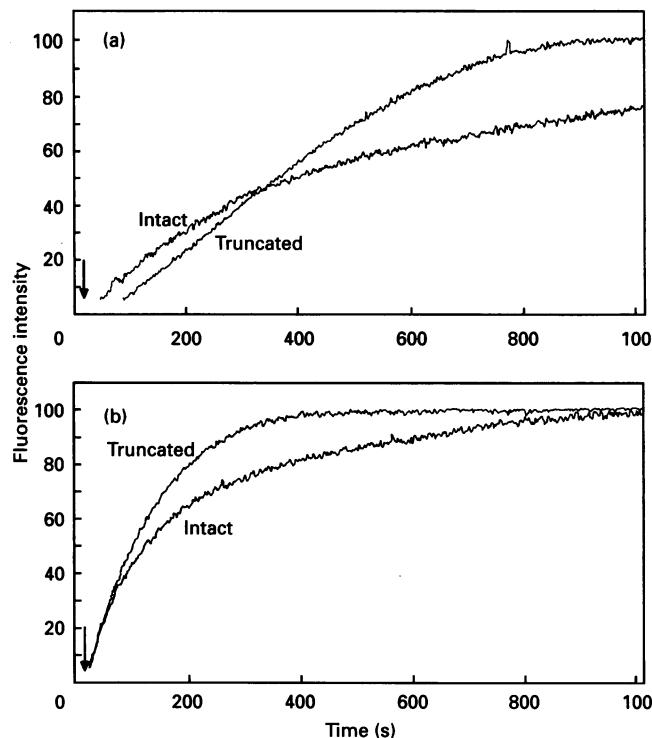


Figure 5 Comparison of the kinetics of polymerization of truncated and intact actin

Solutions of 12 µM (a) or 24 µM (b) actin in buffer G containing 50 µM CaCl₂ were pre-incubated with 0.2 mM EGTA/0.1 mM MgCl₂ and 0.1 µM NBD-labelled intact G-actin for 10 min at 25 °C. Polymerization was then started by addition of KCl at a final concentration of 20 mM (arrow) and the increase in the intensity of NBD fluorescence was recorded. The fluorescence curves were normalized to 0 at time zero and 100 when the steady-state plateau value was reached.

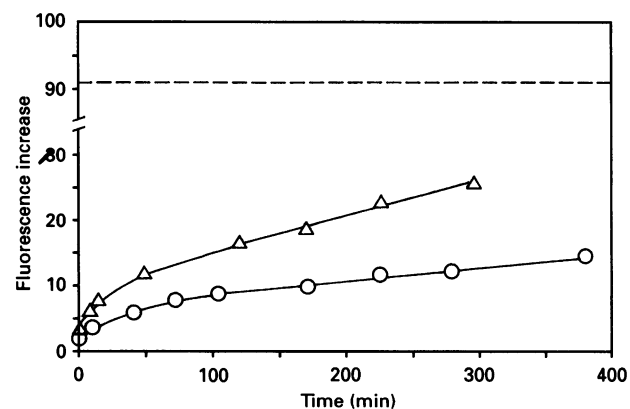


Figure 6 Steady-state exchange of subunits in KCl-polymerized actins

Mg-G-actins were polymerized at a protein concentration of 100 µM and then diluted with a Ca²⁺- and ATP-free polymerization buffer. The exchange reaction was initiated by addition of eATP when the new steady-state level of polymerization, monitored by measuring the 90° light-scattering intensity, was established. The reaction mixtures contained 5 µM truncated (Δ) or intact (○) actin, 4 mM Hepes, pH 7.6, 5 µM CaCl₂, 100 µM EGTA, 50 µM MgCl₂, 0.1 M KCl, 11 µM ATP and 100 µM eATP. The temperature was kept at 25 °C. The differences between the fluorescence intensity of eATP in the reaction mixtures and that of 100 µM eATP alone are plotted in the figure. The broken line shows the equilibrium value for similarly treated non-polymerized actins.

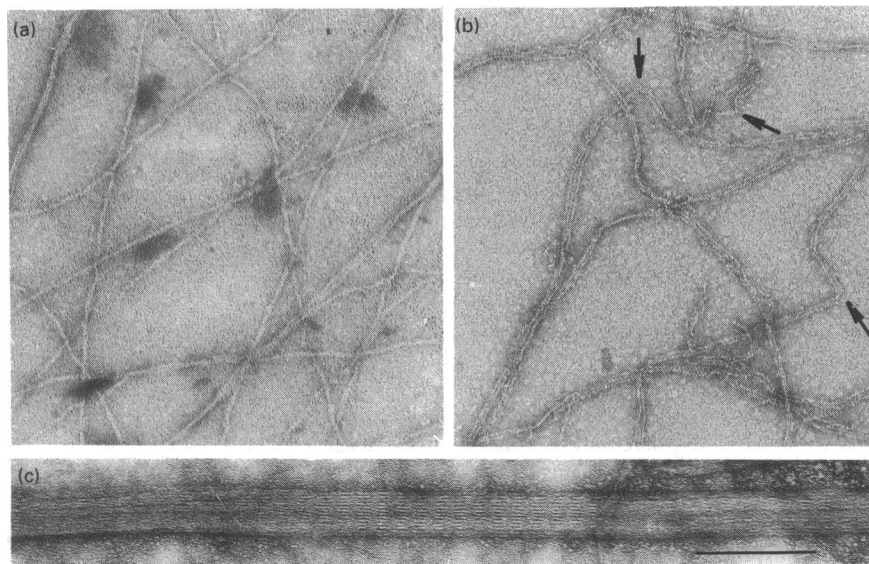


Figure 7 Electron micrographs of truncated and intact actin

(a) and (b) show intact and truncated Mg-actin respectively, polymerized with 0.1 M KCl for 1 h. Points where filaments or filament bundles seem to break are indicated by arrows. (c) Paracrystal of truncated actin formed in 20 mM CaCl_2 . Scale bar, 200 nm.

of the modified and intact actin the same (Figure 5b). A pattern similar to that in Figure 5(b) was also obtained when polymerization of 9.5 μM actin was initiated by addition of KCl together with 1.5 μM F-actin as a source of polymerization nuclei (results not shown). These observations demonstrate that removal of the C-terminal residues diminishes the rate of nucleation.

Instability of the filaments of truncated actin

In the experiment shown in Figure 6 the steady-state exchange of F-actin subunits was studied by measuring exchange of ϵ -ATP with F-actin-bound nucleotides [35]. This method makes use of the increase in the fluorescence intensity of ϵ -ATP upon its binding to actin [36]. The initial fast phase corresponds to exchange of actin-bound nucleotides in the monomer pool and equilibration of labelled monomers with F-actin subunits close to polymer ends. The subsequent slow increase in fluorescence reflects the exchange of internal subunits through constant head-to-tail polymerization (treadmilling) and/or random fluctuations in the filament lengths [35,37,38]. In agreement with earlier observations in KCl-polymerized intact actin the monomer exchange after the initial fast phase was strongly suppressed. In truncated actin the fluorescence continued to increase at a rate that was significantly higher, indicating that removal of the C-terminal residues accelerates the monomer dissociation from one or both polymer ends, since monomer dissociation is rate limiting for the nucleotide exchange in this phase. More extensive spontaneous fragmentation of the filaments of truncated actin, resulting in an increase in the number of filament ends, would also contribute to the observed difference in the kinetics of nucleotide exchange between the two actins. Comparison of the negatively stained preparations of truncated and intact F-actin (Figure 7) suggests that, indeed, the filaments of truncated actin are more prone to break. A similar effect due to removal of only two C-terminal residues has been reported by O'Donoghue et al. [8]. Assembling of the filaments of truncated actin into

paracrystals at high concentrations of MgCl_2 or CaCl_2 had a stabilizing effect (Figure 7c).

Extensive lateral association of the filaments is another common property of actins which lack either three (this work, Figure 7b) or two [8] C-terminal residues. Exposure of the basic residues 371–372 or 371–373 respectively, facilitating their electrostatic interactions with acidic residues on another filament, is a likely explanation for the increased extent of filament cross-linking in the preparations of C-terminal-truncated actins. This possibility was also discussed by O'Donoghue et al. [8].

DISCUSSION

The results presented in this paper show that one can easily obtain homogeneous preparations of actin devoid of the three C-terminal residues (373–375) by digestion of Mg-G-actin with trypsin. We also show that limited tryptic digestion of F-actin results in a mixture of polypeptide chains of residues 1–373 and 1–372, although the contribution of the latter species can be minimized by controlling the removal of Cys-374.

Removal of the three-residue C-terminal segment appears to influence significantly the monomer–monomer interactions. The critical concentration for polymerization of truncated actin is about 5-fold higher than that of intact actin. The opposite effect of removal of only two C-terminal residues has been reported by O'Donoghue et al. [8], based on measurements of copolymerization of the modified actin with pyrenyl-labelled intact actin. Since this assay is inappropriate for analysis of the critical concentration, when the polymerization properties of the copolymerized species are different [39], the possibility that polymerization of actin is influenced differently by removal of either two or three C-terminal residues requires further examination. Studies on polymerization of actin devoid of only the C-terminal phenylalanine residue have led to contradictory results [5,6].

The increase in critical concentration, upon removal of the three-residue C-terminal segment, is consistent with the en-

hancement of the rate constants for monomer dissociation from the polymer ends which is apparent from measurements of the steady-state subunit exchange in F-actin solutions. The enhancement of the rate constants for the dissociation reactions would also explain the increased dynamics of Mg^{2+} -induced oligomers, resulting in the acceleration of ATP hydrolysis in low-ionic-strength solutions, as well as the lower rate of nucleation of truncated actin in polymerizing conditions. The higher overall rates of polymerization of the modified actin seem to be related to more extensive spontaneous fragmentation of the filaments assembled from this actin, the process providing additional polymer ends at which monomers can add. Enhanced fragmentation of actin filaments has also been observed after certain modifications of Cys-374 [40,41].

It remains to be established whether, and in what manner, the increased tendency of the filaments of C-terminal-truncated actins towards lateral association, observed here and by others [8], influences the polymerization of these actins. As mentioned in the Results section one type of the glutaraldehyde-fixed Mg^{2+} -induced actin oligomers, the dimer with apparent molecular mass of 86 kDa, is likely to correspond to the 1,4-PBM-cross-linked LD of intact actin, most probably involving interfilament-type interactions, whereas the 115 kDa species might correspond to the 1,4-PBM-cross-linked UD, representing an intrafilament type of interaction between subunits [31]. The higher ratio of the 86 kDa to 115 kDa species in the solutions of truncated actin correlates with more extensive lateral association of the filaments of this species compared with intact actin. This correlation suggests that interfilament-type interactions may indeed play a role in Mg^{2+} -dependent oligomerization of actin in low-ionic-strength solutions. Since isolated LD of intact actin was shown to be unable to nucleate the polymerization [31], formation of this type of oligomer at the expense of the polymerization-competent UD might contribute to a lowering of the rate of polymerization of truncated actin. On the other hand, as discussed by Millonig et al. [31], one can suppose that the LD can be incorporated into nuclei or growing filaments through one of its subunits and, under conditions favouring filament bundling (e.g. at high protein concentrations), initiate elongation of filament doublets. C-terminal-truncated actins seem to be good material with which to investigate this possibility.

Precise location of residues 373–375 in the monomer structure is still uncertain [4]. Based on model building, Holmes et al. [1] have predicted that the C-terminal phenylalanine residue can participate in the intermonomer interactions along the two-start actin helix. The results presented in this work support their conclusion and point to the importance of this interaction for stabilization of both the polymer and the polymerization-competent oligomers.

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