Long-range conformational effects of proteolytic removal of the last three residues of actin

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Truncated derivatives of actin devoid of either the last two (actin_{-2C}) or three residues (actin_{-3C}) were used to study the role of the C-terminal segment in the polymerization of actin. The monomer critical concentration and polymerization rate increased in the order: intact actin < actin_{-2C} < actin_{-3C}. Conversely, the rate of hydrolysis of actin-bound ATP during spontaneous polymerization of Mg-actin decreased in the same order, so that, for actin_{-3C}, the ATP hydrolysis significantly lagged behind the polymer growth. Probing the conformation of the nucleotide site in the monomer form by measuring the rates of the bound nucleotide exchange revealed a similar change upon removal of either the two or three residues from the C-terminus. The C-terminal truncation also resulted in a slight decrease in the rate of subtilisin cleavage of monomeric actin within the DNase-I binding loop, whereas in F-actin subunits the susceptibility of

this and of another site within this loop, specifically cleaved by a proteinase from *Escherichia coli* A2 strain, gradually increased upon sequential removal of the two and of the third residue from the C-terminus. From these and other observations made in this work it has been concluded that perturbation of the C-terminal structure in monomeric actin is transmitted to the cleft, where nucleotide and bivalent cation are bound, and to the DNase-I binding loop on the top of subdomain 2. Further changes at these sites, observed on the polymer level, seem to result from elimination of the intersubunit contact between the C-terminal residues and the DNase-I binding loop. It is suggested that formation of this contact plays an essential role in regulating the hydrolysis of actin-bound ATP associated with the polymerization process.

INTRODUCTION

On the basis of the atomic model of F-actin, Holmes et al. [1] have suggested that the C-terminal phenylalanine (Phe³⁷⁵), located in subdomain 1 of the actin molecule [2], interacts with the N-terminal part of the DNase-I-binding loop (residues 39–51) on the top of subdomain 2 in the neighbouring polymer subunit along the two-start F-actin helix. The exact location of the C-terminal segment in both the monomer and polymer structure is, however, still uncertain. The three C-terminal residues of actin were proteolytically removed prior to crystallization of the actin–DNase I complex that was used by Kabsch et al. [2] to obtain the first atomic model of G-actin. In the two other atomic models of G-actin, based on the crystal structure of gelsolin segment 1–actin complex [3] and of the profilin–actin complex [4], the position of the C-terminal residues might be changed by their interaction with segment 1 of gelsolin and with profilin respectively.

The role of the C-terminus in actin–actin interactions has been studied using actin modified at the penultimate cysteine residue (Cys³⁷⁴) with certain thiol compounds [5,6], truncated actin lacking either the last two [7,8] or three amino acid residues [9], and mutant β -actin from yeast in which Cys³⁷⁴ was replaced with a serine residue [10]. All these modifications resulted in destabilization of the actin filament.

To obtain more information on the involvement of the C-terminal segment of the actin polypeptide chain in polymer formation and stabilization, we have directly compared certain aspects of polymerization of truncated actin derivatives devoid of the last two (actin_{-2C}) and the last three residues (actin_{-3C}).

Influence of removal of the C-terminal residues on the conformation of other regions of the actin molecule in the monomer and polymer form was investigated by the method of limited proteolysis and by measuring the rates of dissociation of the bound nucleotide and of the tightly bound bivalent cation. The results show that the influence of the proteolytic modifications of the C-terminal segment on polymer formation and its structure is more complex than might be expected from elimination of the putative actin–actin contact made by Phe³⁷⁵ only. Evidence is presented showing that structural perturbations in the C-terminal segment are propagated to distant regions of the monomer molecule, and that proteolytic removal of Lys³⁷³ in addition to Cys³⁷⁴–Phe³⁷⁵ has additional long-range conformational effects on the polymer subunits.

Some of the present results have been presented in a preliminary form [11].

MATERIALS AND METHODS

Protein preparations

Rabbit skeletal-muscle actin was prepared as described by Spudich and Watt [12]. Both intact actin and its C-terminal truncated derivatives were stored in buffer containing 2 mM Hepes, pH 7.6, 0.2 mM ATP, 0.1 mM CaCl₂ and 0.02 % NaN₃ (buffer G). In all experiments on actin (intact or modified) containing Mg²⁺ at the single high-affinity site for bivalent cation, Ca-G-actin was transformed into Mg-G-actin directly before starting the measurements, by a 5–10 min incubation with 0.2 mM EGTA/50 μ M MgCl₂ at room temperature.

Abbreviations used: 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; AEDANS, acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; ϵ -ATP, 1-*N*⁶-etheno-ATP; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulphonyl fluoride; ECP, *Escherichia coli* A2 strain proteinase (EC 3.4.21.62).

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Labelling of actin at Cys³⁷⁴ with *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine (1,5-IAEDANS) was performed as described in [13].

Actin_{-2C} was prepared essentially as described in [7]. Mg-G-actin was polymerized with 0.1 M KCl and digested with trypsin at an enzyme/protein mass ratio of 1:20 at 25 °C. To minimize the proteolytic removal of the third residue (Lys³⁷³) from the C-terminus [9], F-actin labelled with 1,5-IAEDANS was used and the removal of Cys³⁷⁴ was monitored by measuring the decrease in the fluorescence intensity of the AEDANS label. As soon as the fluorescence plateau was reached, proteolysis was terminated by addition of soybean trypsin inhibitor (SBTI) at three times the trypsin concentration. The modified actin was collected by ultracentrifugation and further freed of residual trypsin and low-molecular-mass digestion products by an additional polymerization-depolymerization cycle.

Actin_{-3C} was prepared by digestion of ATP-Mg-G-actin with trypsin as described previously [9]. Tryptic removal of Lys³⁷³ from this form of actin was shown to proceed at a rate 2–3-fold lower than the rate of removal of the Cys³⁷⁴-Phe³⁷⁵ dipeptide [9]. To ensure that all three residues were cleaved off, the release of Cys³⁷⁴ was monitored as described for actin_{-2C}, using actin labelled with 1,5-IAEDANS, and the digestion was carried out for a period of time 3-fold longer than that during which the fluorescence of the AEDANS label decreased to the plateau value. The digestion was stopped with SBTI, and truncated actin was purified by polymerization with 0.1 M KCl/2 mM MgCl₂ and ultracentrifugation, followed by depolymerization by dialysis against buffer G.

G-actin containing 1-*N*⁶-etheno-ATP (ϵ -ATP) at the nucleotide site (ϵ -ATP-G-actin) was obtained by homogenization of centrifuged F-actin pellets in buffer G containing ϵ -ATP in place of ATP, followed by dialysis against the same buffer solution.

All protein preparations, after dialysis, were clarified by a 30 min centrifugation at 150 000 *g*.

Protein concentration in G-actin solutions was determined spectrophotometrically at 290 nm using an absorption coefficient (ϵ) of 0.63 mg·ml⁻¹·cm⁻¹ [14]. Molar concentrations of ATP and ϵ -ATP were calculated using absorption coefficients of 15 400 M⁻¹·cm⁻¹ at 259 nm and 5700 M⁻¹·cm⁻¹ at 265 nm respectively.

Assays

Polymerization of actin was monitored by recording the increase in the light-scattering intensity at 90 ° at 360 or 450 nm, in either a Hitachi 650-10S or a Perkin-Elmer LS-5b luminescence spectrometer.

The critical concentrations of actin for polymerization were determined by measuring the concentration of unpolymerized actin in steady-state F-actin solutions using the DNase-I inhibition assay [15]. Actin in buffer G was polymerized with 0.1 M KCl for 1.5–3 h at high protein concentration. It was then diluted with buffer G supplemented with 0.1 M KCl to several different protein concentrations over the range 5–10 μ M. The samples were incubated at 22 °C until the new steady-state level of polymerization (as monitored by measuring the 90 ° light-scattering intensity) was established, and then the DNase-I inhibitory activity was determined under standard conditions as described in [16] using DNase-I dissolved in buffer G supplemented with 1 mM phenylmethanesulphonyl fluoride (PMSF) and dialysed overnight against this solution. The reaction was started immediately after mixing DNase I and actin, and the DNase-I activity was obtained from the slope of the linear part of the curve of DNA hydrolysis. The concentration of unpoly-

merized actin was calculated from calibration curves of the DNase-I activity versus known amounts of monomeric actin.

ATP hydrolysis associated with actin polymerization was assayed in aliquots removed at time intervals from the cuvette in which the light-scattering intensity of polymerizing actin solution was being measured. The reaction was quenched by mixing the solution with an equal volume of ice-cold solution of 0.6 M perchloric acid, and P_i was determined by the highly sensitive Malachite Green method described in [17].

The dissociation rates of G-actin-bound nucleotide were determined by displacing the initially bound nucleotide, ATP or ϵ -ATP, with a large molar excess (10–100-fold) of ϵ -ATP or ATP respectively, so that the back reaction could be neglected. Unless stated otherwise, concentrated actin solution (about 70 μ M) in buffer G was diluted to the final concentration of 3.5 μ M (ATP-G-actin) or 0.4 μ M (ϵ -ATP-G-actin) with a buffer solution containing 4 mM Hepes, pH 7.6, and either CaCl₂ at different concentrations or 0.2 mM EDTA, and the reaction was immediately started by addition of 0.1 mM ϵ -ATP or ATP respectively. Displacement of the bound nucleotide was monitored by measuring the changes in the fluorescence intensity of ϵ -ATP on its binding to, or dissociation from, actin [18].

The rates of dissociation of Ca²⁺ from the high-affinity site in G-actin were measured using the fluorescent calcium chelator Quin-2 [19]. Actin in buffer G (70 μ M) was 3-fold diluted with a Ca²⁺-free buffer G, then a mixture of Quin-2 and MgCl₂ at final concentrations of 0.5 mM and 0.1 mM respectively was added and the increase in the fluorescence of Quin-2 was recorded at 23 °C. The rate of Ca²⁺ dissociation from actin was calculated from the time course of the slow increase in the fluorescence following the initial fast change corresponding to binding to Quin-2 of free Ca²⁺.

Proteolytic digestions of G- and F-actins were carried out at 25 °C at a protein concentration of 24 μ M. Unless stated otherwise, the digestion mixtures contained 2 mM or 10 mM Hepes, pH 7.6 (for G- and F-actin respectively), 0.2 mM ATP, 0.1 mM CaCl₂, 0.02 % Na₂S₂O₃ and, when present, 0.1 M KCl. In experiments on F-actins the polymerization was controlled by measuring the increase in light-scattering intensity, and the digestions were started shortly after the steady-state value was reached. Digestion with subtilisin and trypsin was stopped with PMSF (2 mM) or SBTI (3 × molar concentration of trypsin) respectively. Activity of the proteinase from *E. coli* A2 strain was quenched by mixing with electrophoresis sample buffer [1.5 % SDS/3 % 2-mercaptoethanol/15 % glycerol/40 mM Tris, pH 6.8 (all final concns.)] and incubation for 3 min at 100 °C. The digests were analysed by SDS/PAGE. Absorbance of Coomassie Blue-stained protein bands on the gels was determined with a Shimadzu CS-9000 dual-wavelength flying-spot scanner.

Other methods

Fluorescence measurements were performed in either a Perkin-Elmer LS-5b luminescence spectrometer or a Hitachi 650-10S spectrofluorimeter. Excitation and emission wavelengths were 340 and 460 nm respectively for AEDANS-actin, 350 and 410 nm for ϵ -ATP and 340 nm and 500 nm for Quin-2. SDS/PAGE was carried out as described by Laemmli [20] on 10 %-(w/v) polyacrylamide gel slabs.

Reagents

Trypsin (from bovine pancreas, tosylphenylalaninechloromethane-treated), subtilisin (Carlsberg; from *Bacillus licheniformis*), ATP (disodium salt), ϵ -ATP (sodium salt), DNase-I

(from bovine pancreas), Quin-2 and EGTA were purchased from Sigma. *E. coli* A2 strain proteinase was a gift of Dr. S. Yu. Khaitlina and A. Morozova (Institute of Cytology, St. Petersburg, Russia). 1,5-IAEDANS was from Molecular Probes. Hepes and SDS were from Serva.

RESULTS

Polymerization properties of intact and C-terminal truncated actins

We have previously shown that ATP-Mg-G-actin devoid of the last three residues has a 5-fold higher critical concentration but polymerizes faster than intact ATP-Mg-G-actin [9]. Figure 1 shows that the rate of polymerization of actin_{-2C} is intermediate between those of actin_{-3C} and of intact actin. To reveal better the differences, for this experiment the conditions of slow polymerization (low salt concentration) were chosen.

The critical concentration for 0.1 M KCl-induced polymerization of actin in ATP-Ca-form, determined using the DNase-I inhibition assay [15], was 1.3 μ M for intact actin, 2.7 μ M for actin_{-2C}, and 3.5 μ M for actin_{-3C}. These measurements were confined to actins with Ca²⁺ as the tightly bound cation, because truncated Mg-F-actins were too quickly depolymerized by DNase I, as indicated by increase in the rate of DNA hydrolysis during the assay. This was not observed with Ca-F-actins. To minimize possible errors due to the protein inactivation, the measurements were performed on freshly obtained preparations only. As reported previously for actin_{-3C} [9], the truncated actins in their monomer form were fairly stable provided they were kept in Ca²⁺-bound form. It was checked, by measuring the DNase-I activity as a function of G-actin concentration, that the truncated actins do not differ from intact actin in their DNase-I inhibitory activity (results not shown).

Polymerization of ATP-G-actin is accompanied by hydrolysis of the bound nucleotide to ADP. It has been established that ATP is hydrolysed subsequent to polymer formation, so that a transient ATP-F-actin species is formed and, in the presence of free ATP, a cap of ATP-subunits is present at least at one, the fast-growing, end of the filament at steady state (for a review, see [21]). With Mg-actin, the uncoupling of the hydrolysis from polymer formation can, however, be observed only when the rate of elongation is very high, i.e. at high actin concentrations, under conditions of 'seeded' polymerization additionally accelerated

Figure 1 Comparison of the kinetics of polymerization of unmodified and C-terminal truncated actins

Actin [12 μ M in 2 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl₂] was preincubated with 50 μ M MgCl₂/200 μ M EGTA for 10 min at 25 °C to replace the tightly bound Ca²⁺ with Mg²⁺. Polymerization was then started by addition of KCl at a final concentration of 10 mM (arrow) and the increase in the light scattering intensity at 360 nm was recorded. Curves 1–3 refer to unmodified actin, actin_{-2C}, and actin_{-3C} respectively. Abbreviation: a.u., arbitrary unit.

Figure 2 Time courses of spontaneous polymerization of unmodified and C-terminal truncated ATP-Mg-G-actins and of the accompanying hydrolysis of ATP

Unmodified actin (a), actin_{-2C} (b) and actin_{-3C} (c), 24 μ M, in 2 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl₂, were preincubated with 0.1 mM MgCl₂/0.2 mM EGTA for 10 min at 20 °C and then polymerized by the addition of KCl at a final concentration of 90 mM (zero time). $\circ$, Light-scattering intensity at 360 nm, normalized to 0 at zero time and 100 at steady state (the results are means for three different preparations; the vertical bars represent the S.D.); $\square$, $\triangle$, ∇ , hydrolysed ATP, determined as described in Materials and methods section; the different symbols denote the results of the three separate experiments.

by sonication [22]. As Figure 2(c) shows, in contrast with intact actin, spontaneous polymerization of the ATP-Mg-form of actin_{-3C} was characterized by a long delay in ATP hydrolysis relative to the polymer formation. Only about 0.3 mol of ATP/mol of actin was hydrolysed at the time when the light-scattering intensity reached the steady-state value. This strong uncoupling of the two reactions could not be simply due to faster polymerization of actin_{-3C} as compared with intact actin, because the hydrolysis on actin_{-3C} was significantly delayed also in relation to ATP hydrolysis on intact actin. The hydrolysis on polymerizing actin_{-2C} (Figure 2b) lagged behind the polymer formation too, but significantly less than in actin_{-3C}. The results obtained for actin_{-3C} also show a strong co-operativity of ATP hydrolysis, supporting the earlier conclusion that the hydrolysis on an ATP-F-actin subunit is accelerated by interaction with ADP-F-actin subunits [22].

Influence of the C-terminal residues on the conformation of the nucleotide site in G-actin

To obtain information on possible differences in the conformation of the nucleotide site in the monomeric form of intact and truncated actins, we have compared the rates of dissociation of the bound nucleotide from these actins making use of a large

Figure 3 Time courses of exchange of G-actin-bound ATP with ϵ -ATP

G-actin ($3.5 \mu\text{M}$ in 4 mM Hepes (pH 7.6)/ $5 \mu\text{M}$ CaCl_2 / $10 \mu\text{M}$ ATP) was preincubated with $50 \mu\text{M}$ MgCl_2 / $100 \mu\text{M}$ EGTA for 10 min at 23°C . At zero time, ϵ -ATP was added to a final concentration of $100 \mu\text{M}$, and its exchange with the actin-bound ATP was monitored by measuring the increase in the fluorescence intensity as described in Materials and methods section. F_∞ is the fluorescence intensity at equilibrium, F_t is the fluorescence intensity at time t , and F_0 is the fluorescence intensity of ϵ -ATP alone. $\circ$, Unmodified actin; $\triangle$, actin₂₀; $\square$, actin₃₀.

Figure 4 Time courses of exchange of the bound nucleotide with ϵ -ATP in F-actin solutions

ATP-Mg-G-actins ($100 \mu\text{M}$ in buffer G additionally containing $50 \mu\text{M}$ MgCl_2 and 0.2 mM EGTA) were polymerized with 0.1 M KCl. The polymerization was monitored by measuring the increase in light-scattering intensity. When steady state was reached, the proteins were 20-fold diluted with a solution containing 10 mM Hepes, pH 7.6, $50 \mu\text{M}$ MgCl_2 , 0.2 mM EGTA and 0.1 M KCl. When the new steady-state level of polymerization was reached, ϵ -ATP was added to the final concentration of 0.1 mM and the exchange reaction was monitored by measuring the increase in the fluorescence intensity of ϵ -ATP as described in the Materials and methods section. The temperature was kept at 25°C . F_0 , F_t and F_∞ denote the fluorescence intensity of ϵ -ATP alone, and those of F-actin solution at time t and at equilibrium respectively. The value of F_∞ was obtained by measuring the fluorescence of $5 \mu\text{M}$ actin polymerized after the nucleotide exchange. $\circ$, Unmodified actin; $\triangle$, actin₂₀; $\square$, actin₃₀.

increase in the fluorescence intensity of an ATP analogue, ϵ -ATP upon its binding to actin [18]. Under identical ionic conditions, in the presence of free Ca^{2+} or Mg^{2+} , the apparent first-order rate constants of ϵ -ATP dissociation from (or incorporation into) the two truncated actin species were the same and higher than that for intact actin (Figure 3). The difference between intact and truncated actins was not large (about 1.5- and 2-fold in the presence of $50 \mu\text{M}$ CaCl_2 and in $50 \mu\text{M}$ MgCl_2 / $100 \mu\text{M}$ EGTA respectively), but well reproducible. It did, however, diminish with decreasing the concentration of free bivalent cation and, in agreement with an earlier report [23], in the presence of $2 \mu\text{M}$ CaCl_2 / $200 \mu\text{M}$ EDTA, it was no longer observed. The rate constants for the release of ϵ -ATP from Ca-G-actin measured under these latter conditions were $(2.55 \pm 0.09) \times 10^{-2} \text{ s}^{-1}$ for intact actin and $(2.45 \pm 0.07) \times 10^{-2} \text{ s}^{-1}$ for actin₃₀ (mean

values $\pm$ S.D.), in good agreement with previously published values [23–25].

The rate-limiting step for the release of G-actin-bound nucleotide is the dissociation of the tightly bound bivalent cation [24,25]. Consistent with this, no difference was also found between the rates of Ca^{2+} dissociation from intact and truncated actins at low free Ca^{2+} , measured with the use of Quin-2 [19]. The first-order rate constants obtained for intact actin and for actin₃₀ were $(3.04 \pm 0.02) \times 10^{-2} \text{ s}^{-1}$ and $(2.89 \pm 0.18) \times 10^{-2} \text{ s}^{-1}$. They are in reasonable agreement with results obtained previously under slightly different conditions [24,25] and are very close to the above-listed values for the rate constants of nucleotide dissociation in the presence of EDTA. In terms of the recently established relationship between the nucleotide and bivalent-cation binding to actin [24–26], taken together these data suggest that removal of the last two or three residues results in lowering of the rate and equilibrium constant for bivalent-cation binding at the high-affinity site in G-actin.

Dynamic properties of the filaments of intact and truncated actins

Our earlier measurements of the kinetics of subunit exchange in F-actin, performed as described by Wang and Taylor [27] by measuring the exchange of ϵ -ATP with F-actin-bound nucleotide, indicated that the steady-state exchange of subunits in the polymer of actin₃₀ is faster than in the polymer of intact actin [9]. As can be seen in Figure 4, the behaviour of the polymer of actin₂₀ is closer to that of intact actin than to actin₃₀. In the present study, the exchange was investigated at a wider time range than employed previously. This enabled us to observe that the differences between the nucleotide exchange rates in the solutions of polymerized actin₃₀ and those of unmodified actin or actin₂₀ are, in fact, confined to the initial period of the fast phase of the fluorescence increase. Within 2–3 h after addition of ϵ -ATP, the exchange rates gradually diminished and eventually decreased to a very low constant rate which was the same for the three actin species.

The properties of the filaments obtained from intact and truncated actins were further investigated using limited proteolysis. G-actin is specifically cleaved between residues 42 and 43 by a proteinase from A2 strain of *E. coli* (ECP) [28], between residues 47 and 48 by subtilisin [29], and the peptide bonds 62–63 and 68–69 are cleaved by trypsin [30]. In the polymer of intact actin, these sites, all located in subdomain 2 of the monomer [2–4], are protected. Proteolysis by subtilisin and trypsin was, however, observed at high enzyme concentrations [31,32]. It seemed to be of particular interest to investigate the effects of removal of the C-terminal residues on the accessibility of the cleavage sites within the surface loop 39–51 in F-actin, since, according to the atomic model of the actin filament [1], this loop is directly involved in the inter-monomer interaction with the C-terminal phenylalanine. As shown in Figures 5 and 6, removal of the last three residues resulted in significant acceleration of the fragmentation of the polymer subunits by both ECP and subtilisin, whereas the rates of digestion of the subunits in F-actin₂₀ were intermediate or closer to those for unmodified F-actin rather than for F-actin₃₀. SDS/PAGE analysis of the digests shows that the patterns of digestion of both intact and truncated F-actins with ECP (Figure 5b) are the same as that reported for unmodified G-actin [28,33]: the 36 kDa C-terminal and about 8 kDa N-terminal fragments (the latter poorly resolved under these electrophoretic conditions) were the only products, indicating that the same peptide bond, between residues 42 and 43, is cleaved. Digestion of actin in both the F- and the G-form with subtilisin, at the high enzyme concentrations used in these

Figure 5 ECP digestion of unmodified and C-terminal truncated F-actins

(a) F-actins, 24 μ M in 10 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl_2 /0.1 M KCl, were digested with ECP at an enzyme/protein mass ratio of about 1:100 at 25 $^{\circ}\text{C}$. Other conditions and procedures were as described in the Materials and methods section. $\circ$, unmodified F-actin; $\triangle$, F-actin_{-2C}; $\square$, F-actin_{-3C}. (b) Electrophoretic patterns of non-digested actin (lane a) and of the 30 min digests of unmodified F-actin (lane b), F-actin_{-2C} (lane c) and F-actin_{-3C} (lane d). Positions of molecular-mass (M) markers (from top to bottom: hen's-egg-white ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; hen's-egg-white lysozyme, 14.4 kDa) and the position of the 36 kDa fragment of actin are indicated.

Figure 6 Subtilisin digestion of unmodified and C-terminal truncated F-actins

(a) F-actins, 24 μ M in 10 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl_2 /0.1 M KCl were digested with subtilisin at an enzyme/protein mass ratio of 1:400 at 25 $^{\circ}\text{C}$. Other conditions and procedures were as described in the Materials and methods section. $\circ$, Unmodified F-actin; $\triangle$, F-actin_{-2C}; $\square$, F-actin_{-3C}. (b) Electrophoretic patterns of non-digested actin (lane a), of the 30 min digests of unmodified F-actin (lane b), F-actin_{-2C} (lane c) and F-actin_{-3C} (lane d), F-actin_{-3C} treated with phalloidin at twice the actin concentration (lane e) and of 1 min digest of unmodified G-actin (lane f). Positions of molecular-mass (M) markers (as in Figure 5) and the position of the 35 kDa fragment of actin are indicated.

experiments, is less specific (Figure 6b). Nevertheless, the digestion patterns clearly show that the accessibility of the peptide bond 47–48, whose cleavage results in the 35 kDa C-terminal fragment [29], was very limited in unmodified F-actin but considerably increased after removal of the C-terminal residues, and that this bond is more easily split in F-actin_{-3C} than in F-actin_{-2C}. As shown for subtilisin (Figure 6b), fragmentation of F-actins by both enzymes was nearly completely inhibited by the presence of phalloidin at twice actin concentration.

The time courses of F-actin cleavage with both ECP and subtilisin were clearly biphasic. The amounts of actin cleaved in

Figure 7 Trypsin digestion of unmodified and C-terminal truncated F-actin

F-actins, 24 μ M in 10 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl_2 /0.1 M KCl were digested with trypsin at an enzyme/protein mass ratio of 1:10 (a) or 1:20 (b) at 25 $^{\circ}\text{C}$. (a) Time course of disappearance of the actin band on SDS-containing gels, determined as described in the Materials and methods section. $\circ$, Unmodified F-actin; $\square$, F-actin_{-3C}. (b) Tryptic removal of the C-terminal dipeptide Cys³⁷⁴-Phe³⁷⁵ from unmodified F-actin, monitored by measuring the changes in the fluorescence intensity of AEDANS-labelled F-actin as described in the Materials and methods section.

the initial fast phase were too large to be accounted for by faster cleavage of the monomer pool. Moreover, quantification of the amounts of actin digested within the initial period of subtilisin digestion of F-actin_{-3C} under conditions such as those described for Figure 6 revealed that the lower, constant, rate of cleavage was established about 15 min after addition of the enzyme (results not shown), whereas the monomeric actin was completely transformed into the 35-kDa C-terminal product within 1 min (Figure 6b). The biphasic character of the cleavage curves could not be also connected with subtilisin cleavage at multiple sites, because the curves for digestion with ECP, which cleaves F-actin subunits at a single site, were biphasic as well. It is plausible that the initial fast phase corresponds to faster digestion of subunits at the filament ends, and mixing of F-actins with enzyme is likely to increase the number of filament ends by fragmentation of the polymers, in particular those of F-actin_{-3C} which in the electron microscope appear more fragile [9].

The above-presented results were obtained with KCl-polymerized ATP-Ca-actins. The same tendencies were observed with KCl-polymerized actins having Mg^{2+} at the high affinity site. The Mg-F-actins were, however, digested at much lower rates. The influence of the kind of bivalent cation bound at the single high-affinity site and at the multiple low-affinity sites [34] on the proteolytic susceptibility of specific sites in F-actin will be described elsewhere (H. Strzelecka-Golaszewska and A. Woźniak, unpublished work).

Fragmentation of F-actin subunits within segment 62–68 by trypsin, generating a 33 kDa C-terminal derivative [30], also tended to be faster in F-actin_{-3C} than in unmodified F-actin (Figure 7a), although here the difference was small. In fact, this experiment compares the rates of fragmentation of F-actin_{-3C} and F-actin_{-2C} rather than intact F-actin, because tryptic removal of the C-terminal dipeptide Cys³⁷⁴-Phe³⁷⁵ from F-actin [7] is fast compared with the cleavages within segment 62–68. This is apparent from Figure 7(b), which shows the time course of the removal of AEDANS-labelled Cys³⁷⁴, monitored by measuring the decrease in the AEDANS fluorescence intensity. At trypsin concentrations twice as low as that used in the experiment illustrated in Figure 7(a), Cys³⁷⁴ was nearly completely removed from KCl-polymerized Ca-G-actin within 5 min.

Figure 8 Subtilisin digestion of unmodified and C-terminal truncated G-actin

(a) G-actins, 24 μ M in the ATP-Mg-form and in 2 mM Hepes (pH 7.6)/0.2 mM ATP/50 μ M CaCl_2 /50 μ M MgCl_2 /0.2 mM EGTA, were digested with subtilisin at an enzyme/protein mass ratio of 1:1500 at 25 °C. Other conditions and procedures were as described in the Materials and methods section. ○, Unmodified G-actin; □, G-actin_{-3C}. (b) Shows electrophoretic patterns of undigested actin (lane a) and of 5 min digests of unmodified actin (lane b) and of actin_{-3C} (lane c).

Conformational coupling between the C-terminal segment and subdomain 2 in monomeric actin

The above-described differences in proteolytic susceptibility of specific sites in subdomain 2 of polymer subunits in F-actins assembled from intact and C-terminal truncated monomers might result from differences in the intersubunit contacts involving this subdomain, from possible differences in the conformation of the polypeptide chain around the cleavage sites in both monomer and polymer subunit, or from a combination of these effects. The digestion experiments on monomeric actins in their Ca^{2+} -bound form have not revealed any significant difference in the rates of cleavage by ECP between unmodified actin and actin_{-3C} (S. Yu. Khaitlina, personal communication). Cleavage with subtilisin between residues 47 and 48 was slightly faster for unmodified G-actin rather than for G-actin_{-3C} (Figure 8). This effect of the removal of the C-terminal residues is opposite to that on the cleavage of F-actin subunits, which suggests that the increased accessibility of the DNase-I-binding loop in C-terminal truncated F-actins is related to gradual weakening of the intersubunit contact between this loop and the C-terminal segment upon removal of the last two and of the third residue. The experiment in Figure 8 also demonstrates that the modification of the C-terminal segment is sensed by the distant region of subdomain 2 in G-actin.

DISCUSSION

The measurements of the critical actin concentrations, polymerization rates and rates of hydrolysis of the bound ATP associated with polymerization presented here show that the polymerization properties of actin gradually change upon successive removal of the last two and of the third residue from the C-terminus. Also, the comparison of structural features of the polymers assembled from intact and truncated actins, such as the rate of subunit exchange and susceptibility of specific sites in F-actin subunits to proteolysis, places actin_{-2C} at position intermediate or closer to unmodified actin rather than to actin_{-3C}. The similarity between actin_{-2C} and unmodified actin can even be larger than it appears from the reported measurements because of contamination of the preparations of actin_{-2C} with actin

devoid of the last three residues. This contamination is inherent in the preparation procedure, which involves tryptic digestion of actin in the F-form. As we have shown previously, polymerization of actin slows down, but does not prevent, the removal by trypsin of the third residue, Lys³⁷³ [9]. So far no simple way to obtain pure actin_{-2C} is known.

The strong uncoupling of ATP hydrolysis from the polymer growth during spontaneous polymerization of the Mg-form of actin_{-3C}, as well as the increased rate of nucleotide exchange in truncated G-actins, suggest that removal of the C-terminal residues perturbs the structure of the neighbouring region of subdomain 1 and that this alteration is transmitted to the cleft where the nucleotide and bivalent cation are bound. This conclusion is supported by the results of recent studies on mutant actins from the fruitfly *Drosophila melanogaster* indicating that certain replacements at positions 362, 364, 366, 368, as well as deletion of the C-terminal residues up to Gly³⁶⁸ slightly reduce (by a factor of up to 1.6) the affinity of actin for ATP [35]. Propagation of conformational changes in the opposite direction, from the cleft to the C-terminal region in subdomain 1 of the monomer, is known from the changes in the fluorescence of the AEDANS label on Cys³⁷⁴ [36,37] and from more recently reported changes in the rate of tryptic removal of the C-terminal residues [33] upon replacement of Ca^{2+} with Mg^{2+} or ATP with ADP (in Mg-G-actin).

Since the nucleotide exchange rate in G-actin was similarly affected by the removal of either the two or the three last residues, there must be additional difference(s) in the polymer structure of actin_{-3C} to account for the strong uncoupling of ATP hydrolysis from the filament elongation observed during polymerization of this actin but not actin_{-2C}. This difference might arise from a change in the intersubunit interactions upon removal of Lys³⁷³, the third residue from the C-terminus. This possibility was tested here by comparing time courses of proteolysis of the three F-actins at specific sites within loop 39–51, the N-terminal part of which has been indicated as the intersubunit contact site for the C-terminal phenylalanine [1]. The rate of cleavage with ECP of the peptide bond between residues 42 and 43 (within the putative contact area for Phe³⁷⁵), as well as that of subtilisin cleavage between residues 47 and 48, significantly increased in the order: control actin < actin_{-2C} < actin_{-3C}. The observed differences are specific for F-actin, since the subtilisin cleavage of the monomer form was slower for actin_{-3C} rather than for unmodified actin. Thus the removal of Lys³⁷³, in addition to the last two residues, seems indeed to weaken the intersubunit contact(s) involving loop 39–51, making it more available to proteolytic enzymes. This conclusion is supported by the observation that phalloidin, which does not directly bind to this loop but is thought to stabilize its contacts with the adjacent subunits [38], strongly protected F-actin_{-3C} from subtilisin and ECP cleavage.

Not only the sites within loop 39–51, but also the distant trypsin cleavage site at Lys⁶⁸ (and, perhaps, at Arg⁶²) in F-actin subunits became more exposed when Lys³⁷³ was removed. It appears, then, that the perturbation of the conformation of the DNase-I binding loop by elimination of its contact with the C-terminal residues, in particular with Lys³⁷³, spreads to other regions of subdomain 2. In a previous study [33] we presented evidence that there is conformational coupling between loop 39–51 and the trypsin cleavage sites at Arg⁶² and Lys⁶⁸ in monomeric actin.

It has been recently shown that substitution of ADP for ATP in Mg-G-actin [33] or binding of P_i or its analogue, beryllium fluoride, at the nucleotide site in ADP-F-actin [32,39] or in ADP-G-actin [32] perturb the conformation of loop 39–51. It is

most probable that the communication between this region of subdomain 2 and the cleft where the nucleotide is bound is bidirectional, so that deviation of this loop from its normal structure in unmodified F-actin, resulting from elimination of its contact with the C-terminal residues of the neighbouring subunit in the growing polymer, is propagated to the nucleotide site, influencing the rate of ATP hydrolysis during polymerization.

Using limited proteolysis it was also possible to detect an allosteric effect of removal of the last three residues on the conformation of loop 39–51 in the monomeric actin. This change was not sensed by DNase I, since no significant difference between intact and truncated actins in their inhibition of DNase-I activity could be observed. This latter observation supports the conclusion that the change(s) in the structure of subdomain 2 on the monomer level do not significantly contribute to the weakening of the intersubunit interactions involving the DNase-I binding loop in F-actin.

On the basis of measurements of the kinetics of incorporation of ϵ -ATP into actin_{3C} and unmodified actin under polymerizing conditions, we have recently suggested that the subunits of the polymer of truncated actin are in a more dynamic state [9]. As we have documented here, the difference in the rates of nucleotide exchange is confined to the initial fast phase of the increase in the fluorescence intensity of ϵ -ATP that corresponds to the nucleotide exchange with actin monomer pool and to monomer exchange at the filament ends. The observed difference could not, however, be due to the faster exchange of the bound nucleotide on monomeric actin_{3C}, because equally fast nucleotide exchange was observed with monomeric actin_{2C}, whereas the behaviour of F-actin_{2C} was closer to that of intact F-actin rather than to F-actin_{3C}. In agreement with the observation by Newman et al. [40], the exchange of F-actin bound nucleotide in the second, linear, phase of fluorescence increase was extremely slow, and there was no measurable difference between the intact and truncated F-actins in this latter phase. This supports the conclusion arrived at by Brenner and Korn [41] that the exchange of polymer subunits mainly occurs through random fluctuations in the length of the polymers, and shows that the very low rates of 'treadmilling' [42] in F-actins assembled from intact and from C-terminal truncated monomers are the same or very similar.

The differences in the monomer critical concentrations of intact and truncated actins obviously contribute to the differences in the exchange rates of their polymer subunits, but do not explain the large difference in the exchange rates between the two truncated actins and very small difference between intact actin and actin_{2C}. Thus, the observed differences in the nucleotide exchange kinetics seem to be due to changes in the rates of both subunit dissociation from, and reassociation with, the polymer ends. It appears that these rates significantly increase upon removal of the third residue from the C-terminus, which supports the conclusion that Lys³⁷³ is somehow involved in the intersubunit interaction.

Taken together, the observations presented here show that there may be multiple ways of communication between the C-terminal segment, the DNase-I binding loop on the top of subdomain 2, and the nucleotide-binding site in the cleft. It is plausible that the allosteric relationship between the nucleotide site and the DNase-I binding loop underlies the hydrolysis of the actin-bound ATP when the conformation of this loop changes upon contact formation with the neighbouring subunits in growing polymer. As has been suggested previously [32,33,39], the release of phosphate may, in turn, bring this loop to yet another conformation. An alternative possibility is that the intersubunit contact formation between the DNase-I binding loop and the C-terminal segment (in intact actin) perturbs the

conformation of this segment, and this change is transmitted through subdomain 1 to the environment of the γ -phosphoester bond of the nucleotide. The direct way of communication between the C-terminal region and the nucleotide site in actin seems to be used by a number of actin-monomer-binding proteins known to either accelerate (profilin [43–46]) or inhibit (β -thymosins [45,46], gelsolin [47]) the exchange of G-actin-bound nucleotide, as the C-terminal segment of the actin polypeptide chain participates in the binding of these proteins [3,4,48].

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