Effects of the type of divalent cation, Ca^{2+} or Mg^{2+} , bound at the high-affinity site and of the ionic composition of the solution on the structure of F-actin

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F-actins containing either Ca^{2+} or Mg^{2+} at the single high-affinity site for a divalent cation differ in their dynamic properties [Carlier (1991) *J. Biol. Chem.* **266**, 1–4]. In an attempt to obtain information on the structural basis of this difference, we probed the conformation of specific sites in the subunits of Mg- and Ca-F-actin with limited proteolysis by subtilisin and trypsin. The influence of the kind of polymerizing salt was also investigated. At high proteinase concentrations required for digestion of actin in the polymer form, subtilisin gives a complex fragmentation pattern. In addition to the earlier known cleavage between Met⁴⁷ and Gly⁴⁸ in the DNase-I-binding loop, cleavage of F-actin between Ser²³⁴ and Ser²³⁵ in subdomain 4 has recently been reported [Vahdat, Miller, Phillips, Muhrad and Reisler (1995) *FEBS Lett.* **365**, 149–151]. Here we show that actually a larger segment, comprising residues 227–235, is removed and the bond between Leu⁶⁷ and Lys⁶⁸ in subdomain 2 is split in both G- and F-actin, and that the differences in the fragmentation patterns of the G- and F-forms are accounted for by the protection of the bond 47–48 in F-actin. The subtilisin and trypsin cleavage sites

in segment 61–69, subtilisin sites in segment 227–235 and trypsin sites between Lys³⁷³ and Cys³⁷⁴ were less accessible in Mg-F-actin than in Ca-F-actin. These are intramolecular effects, as similar changes were observed on $\text{Ca}^{2+}/\text{Mg}^{2+}$ replacement in G-actin. The cation-dependent effects, in particular those on segment 61–69, were however less pronounced in F-actin than in G-actin. The results suggest that substitution of Mg^{2+} for Ca^{2+} , and KCl-induced polymerization of CaATP-G-actin, bring about a similar change in the conformation of subdomain 2 of the monomer. The presence of Mg^{2+} at the high-affinity site also resulted in an increased protection of the bond 47–48. This latter appears to be an intermolecular effect because it is specific for F-actin. The susceptibility to subtilisin and trypsin was also strongly influenced by the kind and concentration of polymerizing salt. The digestion patterns suggest that the exposure and/or flexibility of the regions containing the cleavage sites diminish with enhancement of the ionic strength of the solution. The results are discussed in terms of the current models of F-actin.

INTRODUCTION

A characteristic feature of the F-actin filament is its flexibility. This is apparent from thermal bending motions observed by light microscopy, from the shape of filaments in the electron micrographs, and from various spectroscopic measurements (reviewed in [1]). A number of observations point to the functional importance of this property of actin. Interaction with heavy meromyosin or myosin subfragment 1, in the presence of MgATP^{2-} , increases the thermal bending motions of actin filaments [2]. Filaments assembled from chemically cross-linked monomers are still capable of activating the myosin ATPase activity, but do not move in the motility assay *in vitro* [3,4]. Recent mechanical measurements on isolated filaments [5] and X-ray diffraction studies on muscle [6,7] provided evidence for extensibility of the actin filament, resulting from contraction- or stretch-induced changes in the F-actin helical structure. Involvement of structural flexibility of actin in the generation of force or sliding motion during actin–myosin interaction was suggested long ago by Oosawa and co-workers [1,8,9], and changes in the F-actin helical structure have been suggested to be key events in a model of force generation proposed by Schutt and Lindberg [10].

Flexibility of F-actin has been explained in terms of changes in intersubunit bonding and/or intramonomer conformation [2,10–14]. The latter possibility is consistent with the atomic

structure of the actin monomer, suggesting that the two domains of the molecule as well as their subdomains can undergo motions independently of one another [15–18].

Actin filament flexibility [13,19] and the rate of dissociation/association of monomers at the two ends of the filament (reviewed in [20]) increase significantly on the release of inorganic phosphate that follows the hydrolysis of the actin-bound ATP associated with the filament assembly. Because the release of P_i from F-ADP- P_i -actin is relatively slow, and F-ADP-actin can rebinding inorganic phosphate when present in millimolar concentrations, it is possible that nucleotide-associated processes regulate the mechanical properties and dynamics of actin filaments in the cell (reviewed in [19,20]).

Nucleotide is bound to actin in the cleft separating the two domains of the actin monomer, where it interacts through its phosphate oxygens with a divalent cation bound at the bottom of the cleft [15]. This single high-affinity site for a divalent cation (K_{diss} for Ca^{2+} and Mg^{2+} in the nanomolar range) is most probably occupied by Mg^{2+} *in vivo*. Actin with either Ca^{2+} or Mg^{2+} can easily be prepared *in vitro*, because in monomeric actin this strongly bound cation is easily exchangeable with free divalent cations present in solution. The actin monomer also contains multiple low-affinity sites (reported K_{diss} values for Ca^{2+} and Mg^{2+} in the range 0.1–1 mM) and moderate-affinity sites (K_{diss} for Ca^{2+} and Mg^{2+} in the range 10–100 μM) that can be occupied by both divalent and monovalent cations. Saturation of

Abbreviations used: AEDANS, *N*-acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; Tos-Arg-Met, *p*-tosyl-L-arginine methyl ester hydrochloride.

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the low-affinity sites seems to be the first step in the process of salt-induced actin polymerization *in vitro* (reviewed in [20,21]).

Electron microscopic images of negatively stained filaments and three-dimensional reconstructions calculated from them suggest that the structure and flexibility of the actin filament depend on which divalent cation, Ca^{2+} or Mg^{2+} , is bound at the high-affinity site and also probably on the kind of cation bound at the sites of intermediate affinity [13,14,22]. Other evidence comes from comparison of dynamic properties of Ca-F-actin and Mg-F-actin (references in [20]), measurements of fluorescence anisotropy decay of labelled F-actin [23,24], ^1H NMR spectroscopy [25] and antibody binding studies [26,27].

To obtain more information about the structural changes that could be the basis for changes in flexibility and/or dynamics of the actin filament, we have used limited proteolysis to probe the conformation of specific sites in the actin monomers in Ca- and Mg-F-actin, and their sensitivity to the kind of cation saturating the low- and moderate-affinity sites. The results suggest that, depending on whether the high-affinity site for cation is occupied by Ca^{2+} or Mg^{2+} , there are differences in the structure of actin monomers in F-actin similar to, but less pronounced than, those found with G-actin [28]. We also present evidence that the conformation of several regions of the polymer subunit is sensitive to the ionic strength of the polymerizing solution. The results are discussed in terms of the current models of F-actin.

Some aspects of this study have previously been reported briefly [29].

MATERIALS AND METHODS

Protein preparations

Rabbit skeletal muscle actin, isolated from acetone-dried muscle powder and purified as described by Spudich and Watt [30], was stored in buffer containing 2 mM Hepes, pH 7.6, 0.2 mM ATP, 0.1 mM CaCl_2 and 0.02 % NaN_3 (buffer Ca-G). Ca-G-actin was transformed into Mg-G-actin by a 5–10 min incubation with 0.2 mM EGTA/50 μM MgCl_2 after a 2-fold dilution with Ca^{2+} -free buffer G (composition as above, but without CaCl_2).

Labelling of actin at Cys³⁷⁴ with *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine (1,5-IAEDANS) was performed as described [31].

C-terminal truncated G-actin devoid of the last three residues was prepared by digestion of MgATP-G-actin with trypsin as described previously [32,33].

Protein concentration in G-actin solutions was determined spectrophotometrically at 290 nm, using an absorption coefficient of 0.63 $\text{mg} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1}$ [34]. The concentration of *N*-acetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine-actin (AEDANS-actin) was calculated by the method of Tao and Cho [35].

Proteolytic digestion experiments

All actin digestions were performed at a protein concentration of 24 μM at 25 °C. Ca- and Mg-G-actin were polymerized with either 0.1 M KCl or with 2 mM CaCl_2 or MgCl_2 respectively. Polymerization was monitored by recording the increase in the intensity of scattered light at 450 nm. The digestions were started after the steady-state value of the light-scattering intensity was reached.

To evaluate the influence of various ionic conditions used in the digestion experiments on the intrinsic activity of proteolytic enzymes, the rates of hydrolysis of low-molecular-mass substrates were determined as described previously [28] using 2 mM *p*-tosyl-L-arginine methyl ester hydrochloride (Tos-Arg-Met) for subtilisin (0.24 mg/ml), and 1 mM *N*-benzoyl-L-arginine ethyl ester

hydrochloride for trypsin (10 $\mu\text{g}/\text{ml}$). With subtilisin, no significant difference depending on the kind and concentration of salts present in actin solutions was found. The activity of trypsin was the highest in the buffer containing 2 mM CaCl_2 , decreasing to 90 % and 81 % respectively in the presence of 0.1 M KCl/50 μM CaCl_2 and 0.2 mM EGTA/2 mM MgCl_2 , and to 48 % in the presence of 0.2 mM EGTA/50 μM MgCl_2 /0.1 M KCl (conditions for KCl-polymerized Mg-actin). In the digestion experiments, these differences were compensated for by varying the enzyme concentration.

Digestion with subtilisin was stopped with 2 mM PMSF, and the activity of trypsin was quenched with soybean trypsin inhibitor in 3-fold molar excess. The protein samples were then denatured by incubation for 3 min at 100 °C with electrophoresis sample buffer [1.5 % (w/v) SDS/3 % (v/v) 2-mercaptoethanol/15 % (v/v) glycerol/40 mM Tris, pH 6.8] and subjected to electrophoresis on polyacrylamide gel slabs by the method of Laemmli [36]. The apparent molecular masses of actin fragments were calculated from standard curves obtained with Bio-Rad SDS/PAGE standards (Low Range Kit).

The absorbance of Coomassie Blue-stained protein bands was determined with a Shimadzu CS-9000 dual-wavelength flying-spot scanner. For quantitative comparison of the decay of actin and accumulation of given digestion products, the differences in dye absorption were corrected by dividing the absorbances by the molecular masses of the respective species.

To reveal fluorescent protein bands, the gels were photographed in a UV transilluminator before staining.

Microsequence analysis

The digestion products were separated by SDS/PAGE and then electrophoretically transferred onto a poly(vinylidene difluoride) membrane (Immobilon P) [37] by the conventional tank system. The transfer buffer contained 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (Caps), pH 11, and 10 % (v/v) methanol. Transferred polypeptides were stained with Ponceau S [38] [0.008 %, in 1 % (v/v) methanol/0.4 % (v/v) acetic acid] for 5–10 min. The membrane was rinsed with water and dried in air. Polypeptide bands were excised and sequenced with an Applied Biosystems 470A protein sequencer with program 03RPTH and with an on-line amino acid analyser.

Other methods

Light-scattering intensity and fluorescence were measured in a Perkin-Elmer LS-5b luminescence spectrometer. The excitation and emission wavelengths for AEDANS-actin were 340 and 460 nm respectively.

Reagents

Subtilisin Carlsberg from *Bacillus licheniformis*, trypsin (from bovine pancreas, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one treated), *N*-benzoyl-L-arginine ethyl ester hydrochloride, Tos-Arg-Met, ATP (disodium salt), EGTA and Hepes were purchased from Sigma Chemical Co. SDS was from International Enzymes Ltd., and 1,5-IAEDANS was from Molecular Probes Co.

RESULTS

Sites of subtilisin cleavage in G- and F-actin

Limited digestion with subtilisin is used to study conformational transitions in both monomeric and filamentous actin [28,33,39].

Figure 1 Electrophoretic patterns of subtilisin fragmentation of monomeric and CaCl_2 -polymerized Ca-actin

Actin ($24 \mu\text{M}$ in 2 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.1 mM CaCl_2 and 0.02% NaN_3) was digested, in the monomer form (A) and after polymerization with 2 mM CaCl_2 (B), with subtilisin at an enzyme/protein mass ratio of 1:200 at 25°C . At the times indicated, aliquots of the solutions were withdrawn and the reaction was stopped with PMSF. The digests were analysed by SDS/PAGE on a 15% (w/v) polyacrylamide gel slab. Other conditions were as described in the Materials and methods section.

In G-actin this enzyme preferentially cleaves the peptide bond between Met⁴⁷ and Gly⁴⁸ within the DNase-I-binding loop (residues 38–52) in subdomain 2, producing two fragments with electrophoretic mobilities in SDS/PAGE corresponding to molecular masses of 35 and 9 kDa [40]. At low enzyme-to-protein mass ratios (e.g. 1:1500), the C-terminal 35 kDa fragment is fairly stable [28,40]. At higher enzyme-to-protein mass ratios, such as 1:100 to 1:200, it is rapidly degraded [28,41] to fragments with apparent molecular masses of 33, 25, 19 and 16 kDa (Figure 1A). These fragments, along with the 35 kDa product of cleavage between residues 47 and 48, were also produced, although more slowly, when F-actin was treated with subtilisin at high enzyme-to-protein ratios (Figure 1B). In addition, the F-actin digests contained a 30 kDa fragment that was absent from the digests of G-actin. The results presented below (Figures 4–6) show that the rates of generation of some of these fragments were significantly influenced by the kind of polymerizing salt and/or by the kind of strongly bound cation, indicating that subtilisin might be a good tool for probing metal-ion-dependent conformational transitions in F-actin.

To locate structural changes in F-actin subunits, the as yet unknown subtilisin cleavage sites in actin were identified by sequencing isolated peptides. The digests of Ca- and Mg-F-actin polymerized with CaCl_2 or MgCl_2 respectively were fractionated by SDS/PAGE, and the separated peptides were transferred to a poly(vinylidene difluoride) membrane and subjected to N-terminal sequence analysis as described in the Materials and methods section. Actin specifically labelled at Cys³⁷⁴ with a fluorescent probe, 1,5-IAEDANS [31], was used to identify fragments containing the C-terminus of actin by photographing the gels under ultraviolet illumination before the protein bands were stained with Coomassie Blue. The results are summarized in Table 1.

Additionally, the 25 kDa fragment was cleaved with CNBr and the resulting peptides were subjected to six cycles of automated sequence analysis. The results were consistent with the presence of six CNBr-produced peptides, arising from cleavage at methionine residues at positions 82, 119, 132, 176, 190 and 227. None of the residues following Met²²⁷ in the actin

Table 1 N-terminal sequences and integrity of the C-terminal end of the actin polypeptide chain in products of subtilisin cleavage of F-actin

Fragment (kDa)	N-terminal sequence	Presence of AEDANS-labelled Cys ³⁷⁴
35	Gly ⁴⁸ -Gln-Lys-Asp-Ser-Tyr-Val	+
33	Lys ⁶⁸ -Tyr-Pro-Ile-Glu	+
30	Blocked	—
25	Gly ⁴⁸ -Gln-Lys-Asp-Ser-Tyr	—
19	Lys ⁶⁸ -Tyr-Pro-Ile-Glu	—
16	Ser ²³⁵ -Leu-Glu-Lys-Ser-Tyr-Glu-Leu-Pro	+

Figure 2 Scheme of fragmentation of actin by subtilisin

sequence up to the subtilisin cleavage site between Ser²³⁴ and Ser²³⁵ (Ala-Thr-Ala-Ala-Ser-Ser-Ser) appeared in the corresponding cycles of sequence analysis. The exception was an alanine residue found in cycle 3, but that must have corresponded to Ala¹³⁵. It can be therefore concluded that the whole segment 228–234 is removed by cleavage of the bonds 227–228 and 234–235. It is likely that some other peptide bonds within this segment are cleaved too, as a large part of it is within a surface loop [15–17] containing two alanine residues, and bonds adjacent to alanine are among the preferred sites of subtilisin cleavage.

On the basis of the information from sequence analysis, the identification of fragments containing the C-terminus of actin and the apparent molecular masses of the fragments, the products of subtilisin digestion can be aligned within the primary sequence of actin ([42], with modifications cited in [43]) as depicted in Figure 2. From the digestion patterns shown in Figure 1 we conclude that the fragmentation of both the monomeric actin and F-actin subunits occurs through cleavage of two peptide bonds in subdomain 2, between Met⁴⁷ and Gly⁴⁸ and between Leu⁶⁷ and Lys⁶⁸, and through cleavages within segment 227–235 in subdomain 4 of the molecule. All three sites (regions) are more easily accessible when actin is in the monomeric form. The relative rates of cleavage of G- and F-actin at these places are, however, entirely different. The monomeric form is very rapidly split between Met⁴⁷ and Gly⁴⁸ in the DNase-I-binding loop to yield the C-terminal 35 kDa fragment, whereas proteolysis of the peptide bond 67–68 and degradation of segment 227–235 are slow. This is evident from the delay in the appearance of the C-terminal 33 kDa intermediate product starting with Lys⁶⁸ and of

Figure 3 Changes in the fluorescence of the AEDANS label on Cys³⁷⁴ during fragmentation of F-actin by subtilisin

Ca-G-actin (24 μ M in 10 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.1 mM CaCl₂ and 0.02% NaN₃), labelled at Cys³⁷⁴ with 1,5-IAEDANS, was polymerized with 0.1 M KCl and then digested with subtilisin at an enzyme-to-protein mass ratio of 1:20 at 25 °C in a fluorescence cuvette. The fluorescence intensity of the AEDANS label was recorded as described in the Materials and methods section. Aliquots of the digestion mixture, removed from the cuvette and treated with PMSF to stop the reaction at times indicated by the arrows, were analysed by SDS/PAGE on a 15% (w/v) polyacrylamide gel slab. For other details see the Materials and methods section. (A) The fluorescence record; (B) Coomassie Blue staining (lanes 0 and a–c) and fluorescence profiles (lanes 0' and a'–c') of the polypeptide bands in SDS/polyacrylamide gels. Lanes marked with 0 and 0' show non-digested actin.

the 25, 19 and 16 kDa products of cleavages within segment 227–235, relative to the appearance of the 35 kDa fragment (Figure 1A), and explains the absence of the N-terminal 30 kDa fragment, containing an intact DNase-I-binding loop, from the digests of monomeric actin. In F-actin, in contrast, the bond 47–48 is equally well or, under certain conditions, more strongly protected than segment 227–235, so that the subunits may be cleaved first in this latter region to yield the N-terminal 30 kDa and C-terminal 16 kDa fragments.

It was recently reported that subtilisin digestion of F-actin labelled at Cys³⁷⁴ with 1,5-IAEDANS is accompanied by a decrease in the fluorescence intensity of the label [39]. This observation led the authors to conclude that subtilisin cleaves the F-actin subunits near the C-terminal end. To obtain more information on this cleavage, we supplemented the measurement of changes in the fluorescence of AEDANS-F-actin during digestion with subtilisin with analysis of actin fragmentation patterns and of the fluorescence profiles of the polypeptide bands in samples taken at intervals from the fluorescence cuvette and subjected to SDS/PAGE. Significant changes in the fluorescence of the AEDANS label could be observed only at high subtilisin concentrations, at which F-actin was completely degraded to the intermediates characterized above, which then slowly decayed to small peptides. Figure 3 shows the results obtained at an enzyme-

to-protein mass ratio of 1:20. From a visual inspection of the electrophoretic patterns it seems that the intensities of the fluorescent bands decayed no faster than the intensities of the corresponding Coomassie Blue-stained bands. The C-terminal 35, 33 and 16 kDa fragments present in a sample taken at the fluorescence plateau still contained the fluorescent label. In contrast, there was good correspondence between the magnitude of the decrease in fluorescence intensity measured in the solution (under non-denaturing conditions) and the decrease in the amount of undigested actin calculated from densitometric scans of the gels. In the experiment illustrated in Figure 3, the decrease of the fluorescence intensity after 10 and 30 min of digestion, respectively, was 45% and 74% of the final change. These values compare with a 39% and 72% decrease in the content of undigested actin at these time points. These relationships were reproducibly observed independently of the nature of the strongly bound cation and polymerizing salt. We conclude that there is no specific subtilisin cleavage near the C-terminus of actin, and that the decrease in the fluorescence intensity of the AEDANS label is a long-range effect of fragmentation of F-actin subunits by cleavages within subdomain 2 and/or subdomain 4.

Effects of the nature of the strongly bound cation and of polymerizing salt on the F-actin structure as probed by subtilisin digestion

When MgCl₂ is used to polymerize Ca-G-actin, or, conversely, Mg-G-actin is polymerized by addition of CaCl₂, the cation initially bound at the single high-affinity site is partly replaced by the cation of the polymerizing salt. The extent of replacement depends not only on the different dissociation constants for Ca²⁺- and Mg²⁺-actin but also on the rates of polymerization of these actins, as the polymer formation slows down the exchange. Therefore, to investigate the effects of the type of strongly bound divalent cation on the F-actin structure, Ca-G-actin and Mg-G-actin polymerized with 0.1 M KCl were used. To evaluate the effects of the type of weakly bound cation, a comparison was made between Ca-G-actin or Mg-G-actin polymerized with 2 mM CaCl₂ or MgCl₂ respectively, and the same G-actin species polymerized with KCl. To ensure that the cation-dependent differences in the critical actin concentrations for polymerization [44,45] had no significant influence on the steady-state concentration of the polymer, the experiments were performed at a high concentration of actin (24 μ M). According to the published values for the critical concentrations of Ca- and Mg-actin, the monomer form coexisting with F-actin under our experimental conditions amounted to no more than 2% of total actin.

Figure 4 shows representative fragmentation patterns of Ca- and Mg-F-actin during mild digestion with subtilisin under different ionic conditions. The intrinsic activity of this enzyme, measured with a low-molecular-mass substrate Tos-Arg-Met, was the same regardless of whether polymerization was effected with CaCl₂, MgCl₂ or KCl. The digestion patterns show a strong protection of the peptide bond 47–48 in the DNase-I-binding loop under all conditions used, as the 35 and 25 kDa fragments starting with Gly⁴⁸ appeared in very small amounts. Still lower was the yield of the 33 and 19 kDa fragments originating from cleavage between residues 67 and 68. The 30 and 16 kDa fragments were the major products of subtilisin digestion of CaCl₂- or MgCl₂-polymerized actin (Figure 4, lanes b and e), indicating an exposure of segment 227–235 under these conditions. However, when KCl was used as polymerizing salt, the accessibility of this region too significantly diminished (Figure 4, lanes c and d).

Figure 4 Influence of the kind of strongly bound divalent cation and of polymerizing salt on the pattern of subtilisin digestion of F-actin

G-actins [24 μ M in 10 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.02% NaN_3 and either 0.1 mM CaCl_2 (Ca-G-actin) or 50 μ M CaCl_2 , 0.2 mM EGTA and 0.1 mM MgCl_2 (Mg-G-actin)] were polymerized, in parallel samples, with 0.1 M KCl or with 2 mM CaCl_2 or MgCl_2 . The F-actins were digested with subtilisin at an enzyme-to-protein mass ratio of 1:200 for 1 h at 25 °C. SDS/PAGE was performed on a 10% (w/v) polyacrylamide gel slab. Other details were as described in the Materials and methods section. Electrophoretic patterns of non-digested actin (lane a) and of the digests of CaCl_2 -polymerized Ca-F-actin (lane b), KCl-polymerized Ca-F-actin (lane c), KCl-polymerized Mg-F-actin (lane d) and MgCl_2 -polymerized Mg-F-actin (lane e) are shown. Apparent molecular masses of the digestion products are indicated.

Figure 5 Influence of the kind of strongly bound divalent cation and of polymerizing salt on the pattern of subtilisin digestion of F-actin devoid of the three last residues

Conditions and labelling are as in Figure 4, except that the F-actins were assembled from C-terminal truncated G-actin, prepared as described in the Materials and methods section.

We have previously shown that, in KCl-polymerized Ca-F-actin, the accessibility of the DNase-I-binding loop to proteolytic enzymes increases markedly when the last three residues of actin are cut off [33]. As can be seen in Figure 5, this does not apply to KCl-polymerized Mg-F-actin, whose C-terminal truncated form retained resistance to subtilisin digestion. Comparison of the yields of the 35 kDa product of subtilisin cleavage at Met⁴⁷ in the digests of C-terminal truncated Ca- and Mg-F-actin clearly shows the protection of the DNase-I-binding loop after substitution of Mg^{2+} for Ca^{2+} at the high-affinity site (Figure 5, lanes c and d). The digestion patterns of C-terminal truncated actins also show that the subtilisin cleavage site within the DNase-I-binding loop was more easily accessible in actins polymerized with CaCl_2 or MgCl_2 than in the same species, Ca-F or Mg-F,

Figure 6 Fragmentation patterns of F-actin digested with subtilisin under various ionic conditions at high enzyme concentration

G-actins [24 μ M in 10 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.02% NaN_3 and either 0.1 mM CaCl_2 (Ca-G-actin) or 50 μ M CaCl_2 , 0.2 mM EGTA and 0.1 mM MgCl_2 (Mg-G-actin)] were polymerized, in parallel samples, with 0.1 M KCl or with 2 mM CaCl_2 or MgCl_2 . The F-actins were digested at an enzyme-to-protein mass ratio of 1:20 for 10 min (lanes a, c, e and g) or 30 min (lanes b, d, f and h). SDS/PAGE was performed on a 15% (w/v) polyacrylamide gel slab. Other details were as described in the Materials and methods section. Electrophoretic patterns of the digests of CaCl_2 -polymerized Ca-F-actin (lanes a and b), KCl-polymerized Ca-F-actin (lanes c and d), KCl-polymerized Mg-F-actin (lanes e and f) and MgCl_2 -polymerized Mg-F-actin (lanes g and h) are shown. Apparent molecular masses of the digestion products are indicated.

polymerized with KCl (compare Figure 5, lanes b and c, or e and d).

Figure 6 shows that the increased protection of the DNase-I-binding loop in F-actin having Mg^{2+} at the high-affinity site is not a unique feature of C-terminal truncated actin. Here we took advantage of the relatively fast splitting of unmodified F-actin in all three subtilisin-sensitive regions at high enzyme concentrations. Splitting of the bond between Met⁴⁷ and Gly⁴⁸ was reflected in an accumulation of the 35 and 25 kDa fragments. In KCl-polymerized Mg-actin (Figure 6, lanes e and f), these fragments were produced more slowly than in KCl-polymerized Ca-actin (Figure 6, lanes c and d). Comparison of the fragmentation patterns of KCl-polymerized Ca- and Mg-actin also revealed a protective effect of the strongly bound Mg^{2+} on the two other subtilisin-sensitive regions. Inhibition of cleavage between Leu⁶⁷ and Lys⁶⁸ manifested itself in a lower yield of the 33 and 19 kDa fragments, whereas protection of segment 227–235 is best seen as a decreased amount of the 16 kDa C-terminal fragment. The relatively low yield of the N-terminal 30 kDa product of cleavages within segment 227–235 undoubtedly reflects the enhanced splitting of the peptide bonds 47–48 and 67–68 at high concentrations of subtilisin (note that subtilisin co-migrates with this fragment and, when present in the concentration of 50 μ g/ml used in this experiment, contributes to the density of the 30 kDa band).

The patterns of fragmentation of F-actins at high concentrations of subtilisin also confirm the influence of the ionic composition of the medium on the accessibility of segment 227–235 and of the DNase-I-binding loop and show a similar influence on the cleavage at Leu⁶⁷ (generation of the 33 and 19 kDa fragments). The inhibitory effects of KCl are best seen with Mg-actin (Figure 6, lanes e to h).

As shown in Figure 7 for C-terminal truncated and unmodified Ca-F-actin, the time courses of decay of the actin band on SDS gels during the digestion were clearly biphasic. Assuming that mixing of F-actin solutions with enzyme may result in fragmentation of the filaments and thus increase the number of

filament ends, we have previously suggested that the initial fast phase may reflect faster cleavage of subunits at polymer ends [33]. Our recent observations do not support this conclusion. Time courses of the digestion of F-actin in samples gently mixed with enzyme by tube inversion, or vigorously stirred on an electric shaker for 1, 2 or 3 s, were not significantly different (results not shown). Therefore we consider that the biphasic kinetics of actin decay indicates that the polymer subunits exist in at least two distinct conformational states that differ in proteolytic susceptibility, and that the equilibrium between these states is influenced by the ionic composition of the medium, by the kind of strongly bound cation, and by other modifications of actin (e.g. the removal of the C-terminal residues).

Effects of the nature of the strongly bound cation and of polymerizing salt on the F-actin structure as probed by tryptic digestion

Trypsin is known to cleave G-actin at Arg⁶² and at Lys⁶⁸ in subdomain 2, generating a 33 kDa C-terminal fragment [46]. In F-actin these sites are protected. However, generation of the 33 kDa fragment during trypsin treatment of F-actin was observed at high enzyme-to-protein ratios [33,39]. As can be seen in Figure 8, this fragment appeared in significant amounts in CaCl₂- or MgCl₂-polymerized actins but not when KCl was used as polymerizing agent. Because salt-dependent differences in the intrinsic activity of trypsin were compensated for by varying the enzyme concentration, the increased protection of segment 61–69 in actins polymerized with KCl, in agreement with the observations made with subtilisin as a probe, suggests that the conformation of subdomain 2 in the polymer subunits is highly sensitive to the ionic conditions.

The results in Figure 8 also confirm that segment 61–69 is most strongly protected in KCl-polymerized Mg-actin (compare lanes c and d), although here the influence of the tightly bound cation was largely overshadowed by the inhibitory effect of KCl.

For comparison with F-actin, in Figure 8 (lanes f and g) we also show the patterns of fragmentation of CaATP-G-actin and MgATP-G-actin respectively at similar, high trypsin concentrations. The strong protection of segment 61–69 in G-actin after replacement of the bound Ca²⁺ with Mg²⁺ has been described earlier [28]. Here one can also see that in Mg-G-actin this segment is nearly as resistant to proteolysis as it is in KCl-polymerized actins, so that the effect of polymerization can be seen only with Ca-actin. These results indicate that a significant change in the conformation (or environment) of this area of subdomain 2 occurs during polymerization of Ca-G-actin, and that substitution of Mg²⁺ for the bound Ca²⁺ in G-actin brings about a similar change.

Digestion with trypsin can also be used to probe the conformational transitions in the C-terminal region of actin. In F-actin, this enzyme preferentially cleaves the bond between Lys³⁷³ and Cys³⁷⁴, releasing the C-terminal dipeptide Cys³⁷⁴-Phe³⁷⁵ [32,47]. The time course of this reaction can be followed conveniently by monitoring a decrease in the fluorescence of the AEDANS label on Cys³⁷⁴, as the rate of removal of the C-terminal residues by trypsin is not significantly influenced by the presence of the AEDANS probe [32]. The release of the labelled cysteine residue from F-actin is the only determinant of the fluorescence change during tryptic digestion, as this change is completed before any significant cleavage at the other sites (in subdomain 2) is observed [33]. We have previously reported that substitution of Mg²⁺ for the tightly bound Ca²⁺ slows down the removal by trypsin of the C-terminal residues from KCl-polymerized actin [32]. The results

Figure 7 Time courses of decay of unmodified and C-terminal truncated Ca-F-actins during digestion with subtilisin in the presence of 2 mM CaCl₂

Ca-G-actins (24 μ M in 10 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.1 mM CaCl₂ and 0.02% NaN₃) were polymerized with 2 mM CaCl₂ and then digested with subtilisin at an enzyme-to-protein mass ratio of 1:200 at 25 °C. Aliquots, withdrawn from the reaction mixtures at intervals, were analysed by SDS/PAGE. Relative intensities of actin bands were obtained from densitometric scans of the gel (the band intensity at time = 0 was taken as 100%). For details see the Materials and methods section. Symbols: ○, unmodified F-actin; □, C-terminal truncated F-actin.

Figure 8 Influence of the kind of strongly bound divalent cation and of polymerizing salt on tryptic digestion of F-actin

Ca-F-actins and Mg-F-actins, prepared as described in the legend to Figure 4, were digested with trypsin in parallel with non-polymerized CaATP-G-actin and MgATP-G-actin. The Ca-actin, non-polymerized or polymerized with CaCl₂, was digested at an enzyme-to-protein mass ratio of 1:20. To compensate for the lower intrinsic activity of trypsin under the other ionic conditions, the concentration of enzyme was increased according to the differences in hydrolysis rates observed with a synthetic substrate, *N*-benzoyl-L-arginine ethyl ester hydrochloride (see the Materials and methods section). After 1 h at 25 °C the digestions were stopped with soybean trypsin inhibitor, and the digests were analysed by SDS/PAGE on an 8% (w/v) polyacrylamide gel slab. Electrophoretic patterns of non-digested actin (lane a) and of the digests of CaCl₂-polymerized Ca-F-actin (lane b), KCl-polymerized Ca-F-actin (lane c), KCl-polymerized Mg-F-actin (lane d), MgCl₂-polymerized Mg-F-actin (lane e), CaATP-G-actin (lane f) and MgATP-G-actin (lane g) are shown. Positions of actin, its 33 kDa product, trypsin (Try) and soybean trypsin inhibitor (SI) are indicated.

presented in Figure 9 show that the accessibility of the C-terminal segment to trypsin was reduced, in both Ca- and Mg-F-actin, when KCl rather than CaCl₂ or MgCl₂ was used for polymerization. As in the case of proteolysis at other sites, the removal of the C-terminal residues proceeded in at least two phases, with an initial fast phase affecting about 30% of the F-actin subunits.

Figure 9 Influence of the kind of strongly bound divalent cation and of polymerizing salt on tryptic removal of the two last residues from F-actin

Actin, fluorescently labelled at Cys³⁷⁴ with 1,5-IAEDANS, was polymerized and then digested with trypsin as described in the legend to Figure 8. The digestions were performed in the fluorescence cuvettes, and the release of the C-terminal residues was monitored by measuring a decrease in the fluorescence intensity of the AEDANS label as described in the Materials and methods section. The fluorescence intensities are normalized to 100% at time zero, and to 0% at the final plateau. Symbols: $\square$, $\circ$, Ca-F-actin polymerized with 2 mM CaCl₂ or 0.1 M KCl respectively; $\blacksquare$, $\bullet$, Mg-F-actin polymerized with 2 mM MgCl₂ or 0.1 M KCl respectively.

The salt-dependent effects reflect a sensitivity of F-actin structure to the ionic strength

It is known that K⁺, Ca²⁺ and Mg²⁺ ions compete for the same sites belonging to the classes of low- and moderate-affinity sites

Figure 10 Subtilisin digestion patterns of Ca-actin polymerized with various mixtures of CaCl₂ and KCl

Ca-G-actin (24 μ M in 10 mM Hepes, pH 7.6, containing 0.2 mM ATP, 0.1 mM CaCl₂ and 0.02% NaN₃) was polymerized, in parallel samples, with (a) 2 mM CaCl₂, (b) 1 mM CaCl₂ and 50 mM KCl, (c) 2 mM CaCl₂ and 50 mM KCl, (d) 100 mM KCl, and (e) 2 mM CaCl₂ and 100 mM KCl. The F-actins were digested with subtilisin at an enzyme-to-protein mass ratio of 1:100 for 30 min at 25 °C. The reaction was quenched with 2 mM PMSF, and the digests were subjected to electrophoresis on a 15% (w/v) polyacrylamide gel slab. Densitometric scans of the gel are shown. Apparent molecular masses of the major digestion products are indicated.

in actin, although the binding of K⁺ is much weaker (reviewed in [21]). To induce polymerization of actin, the differences in the binding affinities of these cations are compensated for by using KCl in concentrations higher than those of CaCl₂ or MgCl₂, which leads to large differences in the ionic strength of the solutions. To see whether the observed salt-dependent differences in proteolytic susceptibility of certain sites in F-actin were related to the nature of cation saturating the low- or moderate-affinity sites, or were due to an unspecific effect of the ionic strength, we have compared subtilisin digestion patterns of Ca-G-actin polymerized, in parallel samples, with various mixtures of KCl and CaCl₂. Densitometric scans of the SDS-containing gels are shown in Figure 10. When the concentration of KCl was gradually increased from 0 to 0.1 M and that of CaCl₂ decreased, in the same proportion, from 2 mM to 0 (Figures 10a, 10b and 10d), the intensities of the bands corresponding to the 30, 25, 19 and 16 kDa fragments diminished, consistent with the inhibition of the cleavages within segment 227–235 exerted by KCl. When the KCl concentration was kept constant at 50 mM and that of CaCl₂ was increased from 1 mM (Figure 10b) to 2 mM (Figure 10c), which resulted in a doubling of the Ca²⁺/K⁺ ratio but no appreciable change in the ionic strength of the solution (53 mM in Figure 10b; 56 mM in Figure 10c), there was no significant change in the fragmentation pattern. Similarly, the fragmentation patterns were virtually the same when actin was polymerized with either 100 mM KCl alone (Figure 10d, 100 mM) or with 100 mM KCl supplemented with 2 mM CaCl₂ (Figure 10e, 106 mM). In contrast, increasing the ionic strength of the solution with the Ca²⁺/K⁺ ratio kept constant resulted in a suppression of the generation of fragments with apparent masses in the range 16–30 kDa, resulting from cleavages within segment 227–235. This latter effect is seen when one compares Figure 10b (for 1 mM CaCl₂/50 mM KCl, 53 mM) with Figure 10e (for 2 mM CaCl₂/100 mM KCl, 106 mM). It seems from these results that the salt-dependent differences in actin fragmentation patterns are due to ionic strength effects rather than to saturation of the low- or moderate-affinity sites with either monovalent or divalent cations. Similar experiments with trypsin, reporting the accessibility of segment 61–69, confirm this conclusion (results not shown).

DISCUSSION

There is biochemical [26,28,32,39,48–50] and structural [13,14,22] evidence that actin, in both the monomeric and filamentous forms, assumes different conformations depending on the kind of nucleotide and divalent cation bound with high affinity in the cleft between the two domains of the molecule. Molecular dynamics simulation indicates relative movements of the domains and subdomains of G-actin [18], and results of molecular modelling of Ca-F-actin suggest that polymerization is accompanied by a shift in position of subdomains 2 and 4 whereby the interdomain cleft narrows [51,52]. Narrowing of this cleft on transformation from G-actin to F-actin is also supported by measurements of fluorescence energy transfer between probes on each of the two domains [24].

Replacement of Ca²⁺ by Mg²⁺ in ATP-G-actin was shown to inhibit trypsin cleavage at Arg⁶² and Lys⁶⁸ in subdomain 2, and at Arg³⁷² and Lys³⁷³ in the C-terminal segment [28,32]. The results reported here show similar effects of Ca²⁺/Mg²⁺ exchange on the proteolytic susceptibility of these regions of the molecule in F-actin. However, the influence of changing the divalent cation on F-actin is less pronounced than on the monomer. This could be due to hydrolysis of the bound ATP during polymerization. It is known that conversion of MgATP-G-actin to

MgADP-G-actin diminishes the protective effect of Mg^{2+} on the sites of trypsin cleavage [28]. On the other hand, our present data show a striking similarity of MgATP-G-actin to KCl-polymerized F-actin (with either Mg^{2+} or Ca^{2+} at the high-affinity site) in its resistance to proteolysis within segment 61–69. This finding supports the suggestion [28] that replacement of the tightly bound Ca^{2+} with Mg^{2+} brings subdomain 2 of ATP-G-actin into a conformation more closely resembling that in the F-actin subunit than in the Ca-monomer, and that this change may explain the faster polymerization of MgATP-G-actin than CaATP-G-actin [53]. A similar conclusion was recently arrived at by Kim et al. [54], who probed the conformation of subdomain 2 with actin fluorescently labelled at Gln⁴¹.

The cation-dependent changes cannot yet be described in detail, but could be due to the relative rotation of the two domains of actin around the putative hinge point at Gln¹³⁷, opening or closing the interdomain cleft [18], as the tightly bound divalent cation in actin crystal structures is close to this residue [15–17]. The reduced accessibility to proteolytic enzymes of the polypeptide chain region around Lys⁶⁸, facing the interdomain cleft, might simply be due to narrowing of this cleft. As discussed above, this change was suggested to occur on polymerization of actin. Our results point to the possibility that it also occurs in monomeric actin on substitution of Mg^{2+} for the tightly bound Ca^{2+} .

Relative rotation of the two domains might also underlie the changes seen in the C-terminal segment. In profilin: β -actin crystals [17], where this segment is intact, the C-terminal phenylalanine is buried in a hydrophobic pocket formed by residues from both sides of the small cleft between subdomains 1 and 3 on the other side of the hinge region. Deformation of this cleft, resulting from domain rotation, would also affect the C-terminal region.

The lack of significant influence of Ca^{2+}/Mg^{2+} exchange on the proteolytic susceptibility of segment 42–48 of the DNase-I-binding loop in G-actin [28] argues that the increased protection of the subtilisin cleavage site at Met⁴⁷ in Mg-F-actin as compared with Ca-F-actin is an intermolecular effect. In the current models of F-actin, the DNase-I-binding loop of monomer N makes contact with loops 162–176 and 280–295 in subdomain 3 [51] or with residues in the cleft between subdomains 1 and 3 [52] of subunit N + 2. As discussed above, these areas might be influenced by interdomain movements, possibly owing to Ca^{2+}/Mg^{2+} replacement.

On the basis of the current model of F-actin, Mg^{2+} -induced changes in the actin filament structure seen by electron microscopy have been interpreted in terms of a rotation of subdomain 2 in a plane perpendicular to the filament axis, to a position where the highest-radius contact in the long-pitch helical strand is eliminated [13,14]. If this contact were broken, one would expect cleavage of the DNase-I-binding loop by proteolytic enzymes to be facilitated. The results from digestions of Mg-F-actin and Ca-F-actin with subtilisin show the contrary. One cannot, however, exclude the possibility that the decreased access of the large enzyme molecule to this loop in Mg-F-actin is due to a steric hindrance that appears as a result of cation-dependent conformational transitions in the cleft between subdomains 1 and 3 in subunit N + 2.

We have shown here that, in addition to the cleavage at Met⁴⁷, subtilisin used at high concentrations cleaves both G- and F-actin between Leu⁶⁷ and Lys⁶⁸ in subdomain 2, and within segment 227–235 in subdomain 4. At least two peptide bonds, between Met²²⁷ and Ala²²⁸ and between Ser²³⁴ and Ser²³⁵, are hydrolysed. While this work was being prepared for publication, subtilisin cleavage of F-actin between Ser²³⁴ and Ser²³⁵ was reported by

others [55]. Our results agree with this report and enabled us to demonstrate that conformational transitions in the high-affinity site for cation are also propagated to the outer surface of subdomain 4 of actin.

The protease-sensitive sites in segments 227–235 and 61–69 and near the C-terminus were more easily split in actin polymerized with 2 mM $CaCl_2$ or $MgCl_2$ than in the presence of 0.1 M KCl, with or without divalent salt. Also, the exposure of the DNase-I-binding loop observed in C-terminal truncated F-actins was decreased in the presence of 0.1 M KCl. Analysis of the digestion patterns suggests that these effects reflect ionic-strength-dependent changes in exposure and/or flexibility of the protease-sensitive loops in F-actin subunits. This interpretation is in agreement with recent observation of similar effects on the number of mobile residues in F-actin by means of ¹H NMR spectroscopy [25].

The ionic strength dependence of the configuration or flexibility of several regions of the monomer incorporated into the filament might contribute to the variability of F-actin structure seen by electron microscopic reconstructions of the filaments assembled in different ionic conditions [22]. In particular, the influence of the kind and concentration of polymerizing salt on the C-terminal segment might be important for interpreting the differences in the strength of a bridge of density connecting the opposite long-pitch strands of F-actin. Participation of the C-terminal residues in development of this connectivity was suggested because its strength was inversely correlated with proteolytic susceptibility of the C-terminus, and because it was not seen in C-terminal truncated F-actin [22].

The deviations of the actin decay curves during limited digestion from pseudo-first-order kinetics might reflect different rates of actin splitting at different sites, or an effect of proteolysis at one site on the conformation of other site(s) of cleavage [28]. However, biphasic kinetics has also been observed with an enzyme splitting a single bond in actin [33]. This behaviour supports the suggestion [24,25] that the polymer subunits exist in at least two different conformational states, and the type of the strongly bound cation, ionic strength and perhaps other factors influence the F-actin structure by shifting the equilibrium between these states.

In summary, the biochemical observations suggest that the presence of Mg^{2+} at the high-affinity site facilitates the polymerization of G-actin through long-range conformational changes in the actin molecule that seem to bring subdomain 2 into a conformation similar to that in F-actin and thus favourable for helical polymer formation. Our results also emphasize the role of physiological concentrations of monovalent cations in stabilization of the F-actin structure and call for caution in interpreting the conformational effects observed in non-physiological low-salt conditions.

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