A novel 27/16 kDa form of subtilisin cleaved actin: structural and functional consequences of cleavage between Ser^{234} and Ser^{235}

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Abstract A new 27/16 kDa form of cleaved actin was prepared by subtilisin cleavage between Ser^{234} and Ser^{235} of F(MgADP)-actin complexed with BeF_x. The cleavage had little effect on actin–actin interactions as probed in polymerization measurements and by electron microscopy. In circular dichroism melting experiments the thermostability of F-actin was reduced by about 10°C by this cleavage. The in vitro motility and V_{max}, but not K_m, of actomyosin ATPase were decreased by about 20% upon 27/16 kDa cleavage of F-actin. The binding of tropomyosin to actin was unchanged by this modification.

Key words: Actin; Proteolysis; Actomyosin interaction; Structure–function relationship

1. Introduction

Limited proteolytic digestions are a valuable tool in structure–function studies of proteins. In the monomeric actin (G-actin) three sites located in its subdomains 1 and 2 show general protease susceptibility. Loop 38–52 is cleaved by subtilisin [1], chymotrypsin [2] and *E. coli* protease [3], segment 61–69 is digested by trypsin [4] and chymotrypsin [2], and the C-terminal residues are cleaved by several proteases [2]. Changes in the proteolytic susceptibility of these three sites were elegantly used in a recent study [5] to localize metal ion-and nucleotide-dependent changes in G-actin structure.

The cleavage in the 38–52 loop and the 61–69 segment is inhibited but not blocked by the polymerization of G- to F-actin. Despite this inhibition, tryptic digestion of F-actin yields the same products as that of G-actin, and has been used as a sensitive probe of structural changes in subdomain 2 of actin induced by the binding of P_i and its analog BeF_x to MgADP-F-actin [6]. As reported by Muhlrad et al. [6], BeF_x dramatically and cooperatively inhibited the tryptic cleavage of MgADP-F-actin and also protected it from cleavage in subdomain 2 by subtilisin. These changes are most likely due to the stabilization of subdomain 2 contacts with the adjacent actin unit in F-actin by the bound BeF_x [7]. In this study we describe the formation of a novel 27/16 kDa form of cleaved actin generated by subtilisin digestion of MgADP·BeF_x–F-actin between Ser^{234} and Ser^{235}. This introduces a specific cleavage site into subdomain 4 of actin and provides, for the first time, an experimental tool for probing the role of this subdomain in macromolecular interactions of actin. The main interactions of actin with actin, myosin, and tropomyosin are examined here for the 27/16 kDa actin.

2. Materials and methods

2.1. Preparation of proteins

Actin, tropomyosin, and myosin subfragment 1 (S1) were prepared from rabbit skeletal muscle as previously described [1]. N-terminal and C-terminal anti-actin antibodies were gifts from Dr. G. DasGupta [8].

MgADP·BeF_x–F-actin was prepared as described before [6]. The 27/16 kDa subtilisin cleaved actin was obtained by digestion of MgADP·BeF_x–F-actin (100 μM) with 160 μg/ml of subtilisin Carlsberg (type VIII bacterial protease) at 25°C for 60 min. The reaction was stopped with 1.0 mM PMSF. Subtilisin and free BeF_x were removed from the reaction sample by sedimenting actin and resuspending the pellet in Mg-F-actin buffer (1.0 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM ATP and 5.0 mM Tris-HCl, pH 7.8) and 1.0 mM PMSF. This actin was re-sedimented and depolymerized by dialysis against G-actin buffer (0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP and 5.0 mM Tris-HCl, pH 7.8) and 0.5 mM PMSF. The resulting G-actin was clarified by ultracentrifugation and used in all subsequent experiments.

2.2. Polymerization and structure of actin

The polymerization of intact and 27/16 kDa cleaved actin was followed by light scattering measurements at λ = 325 nm at 4, 15, and 25°C in a Spex Fluorolog Spectrofluorometer [1]. The polymerization of actin (10 μM) was initiated by the addition of 2.0 mM $MgCl_2$. The polymerized actin samples were examined by electron microscopy as previously described [1].

Critical concentrations for the polymerization of intact and 27/16 kDa cleaved actin in G-actin buffer containing 2.0 mM $MgCl_2$ and 0.5 mM PMSF were determined as before [1].

Structural stabilities of intact and cleaved actin were compared in circular dichroism (CD) melting experiments. The elipticities at 222 nm, $[\Theta]_{222}$, of intact and 27/16 kDa cleaved F-actins (4.0 μM), both complexed with MgADP·BeF_x, were measured in Jasco J-600 CD spectropolarimeter during ramp heating of these samples at 1°C/min. These experiments were carried out in 2.0 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM ATP, 5 mM EPPS (pH 7.5), 5 mM NaF and 100 μM $BeCl_2$. The midpoints of unfolding transitions were obtained from derivative plots of $[\Theta]_{222}$ vs. time of heating.

2.3. Functional assays

Actin-activated ATPase activity of S1 and the kinetic parameters V_{max} and K_m were determined as before [6]. The ATPase measurements were carried out in 10 mM KCl, 10 mM imidazole, 3 mM MgATP, 1.0 μM S1 and between 0 and 50 μM actin, at 25°C.

In vitro actin motility assays were performed as previously described [6].

Tropomyosin (Tm) binding to actin was determined by cosedimentation of mixtures of tropomyosin (0.5 and 1.0 μM) and F-actin (5.0 μM) in a Beckman airfuge for 30 min. at 23°C. The pelleted samples were denatured and analyzed by densitometry of Tm and actin bands on SDS-PAGE. Molar ratios of Tm bound to actin were obtained by using appropriate stain calibration gels of known amounts of these proteins.

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Abbreviations: S1; myosin subfragment 1.

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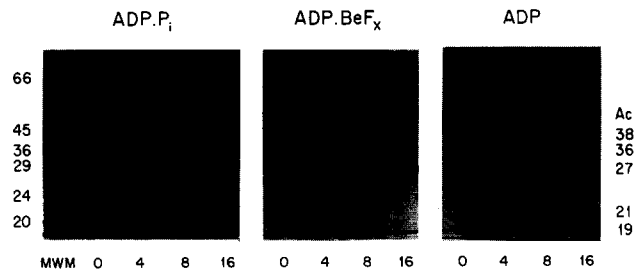

Fig. 1. SDS-PAGE of subtilisin digested F-actin. 20 μM F-actin in 1.0 mM $MgCl_2$, 0.1 mM DTT and 20 mM Tris-HCl, pH 7.8 and 50 μM ATP was digested by subtilisin (40 μg/ml) at 22°C. Sodium phosphate, 20 mM, was present during the digestion when indicated by P_i, and $BeCl_2$ (0.1 mM) and NaF (5.0 mM) were present when indicated by BeF_x. Digestion times and molecular weight standards are shown under the lanes and next to the bands, respectively.

3. Results and discussion

3.1. Formation of a novel 27/16 kDa form of subtilisin-cleaved actin

Subtilisin cleavage of G-actin has been shown to occur with high specificity between Met^{47} and Gly^{48} in subdomain 2, resulting in the formation of two fragments with electrophoretic mobilities corresponding to 36 and 9 kDa masses [1]. Due to strong protection of loop 38–52 in F-actin from proteolysis, subtilisin cleavage of actin filaments requires higher protease concentrations and yields a more complex fragmentation pattern than in G-actin (Fig. 1). At least six proteolytic products with mobilities corresponding to 38, 36, 27, 21, 19, and 16 kDa molecular masses can be identified in the early stages of subtilisin cleavage of F(MgADP)-actin. As previously shown for tryptic cleavage of actin in the 61–69 region [6], addition of phosphate or its analog BeF_x strongly protects F-actin from proteolysis. However, while tryptic digestion of F-actin can be completely blocked by BeF_x [6], its effect on subtilisin cleavage is less complete leaving one site on actin open to protease attack. Consequently, a specific proteolysis of F-actin complexed with BeF_x can be achieved with subtilisin to produce a 27/16 kDa cleaved actin. Under optimal cleavage conditions (see section 2), preparations containing between 80 and 90% of 27/16 kDa actin, can be obtained, uncontaminated by any other degradation products (inset to Fig. 2).

Table 1
Kinetic parameters of acto-S1 ATPase, in vitro motility, and tropomyosin binding to actin

Actin	V_{max} (s^{-1})	K_m (μM)	V_f (μm/s)	Θ (Tm/Actin)
Unmodified	18.2 ± 0.6	6.8 ± 1.0	4.63 ± 0.53	0.08 ± 0.02[a] 0.15 ± 0.01[b]
Cleaved (27/16 kDa)	15.0 ± 0.4	6.0 ± 0.7	3.60 ± 0.84	0.09 ± 0.02[a] 0.16 ± 0.01[b]

V_{max} and K_m values of acto-S1 ATPase, molar ratios of tropomyosin binding to actin (Θ), and mean sliding velocities of actin filaments (V_f) were determined on intact and 85% cleaved actin. V_{max}, K_m, and Θ measurements were made on two separate protein preparations. V_f was measured on four actin preparations, in each case the motion of at least 50 filaments was monitored. Molar ratios of Tm binding to actin (Θ) were determined at 1:10 (a) and 1:5 (b) molar ratios of Tm added to actin. Standard deviations in V_f are given in the table, the standard errors of these measurements were 0.07 and 0.12 μm/s for the unmodified and cleaved actin, respectively.

To identify the 27/16 kDa cleavage site on actin, Western blot analysis of intact and cleaved actin was carried out with N-terminal (1–7) and C-terminal (359–367) actin antibodies [8]. While the intact actin stained with both antibodies, the 27 kDa fragment was only recognized by the N-terminal IgG and the 16 kDa fragment only by the C-terminal antibody (data not shown). This finding enabled Edman N-terminal sequence analysis of the C-terminal 16 kDa actin fragment without its purification from the N-terminally blocked intact actin and the 27 kDa fragment. The first seven residues identified by the sequencing of the 16 kDa fragment were Ser, Leu, Glu, Lys, Ser, Tyr, and Glu placing the 26/16 kDa cleavage site between Ser^{234} and Ser^{235} in subdomain 4 of actin [9]. In analogy to subdomain 2 cleaved actins [1–3], the 27 and 16 kDa fragments were not dissociated from each other in repeated cycles of polymerization and depolymerization of cleaved actin.

3.2. Polymerization and structure of the 27/16 kDa actin

The polymerizations of intact and 27/16 kDa cleaved G-actins (10 μM) by 2.0 mM $MgCl_2$ were compared at 4, 15, and 25°C by light scattering measurements. No significant differences in the rates of their polymerization were detected (data not shown) indicating that the cleavage between Ser^{234} and Ser^{235} in subdomain 4 neither impairs the structural integrity of actin nor does it affect the hydrophobic interactions involved in its polymerization.

The critical concentration for polymerization of actin by 2.0 mM $MgCl_2$ was somewhat increased by the 27/16 kDa cleavage of actin, from 0.2 μM to 0.7 μM for the intact and 85% cleaved proteins, respectively (Fig. 2). This difference did not impact

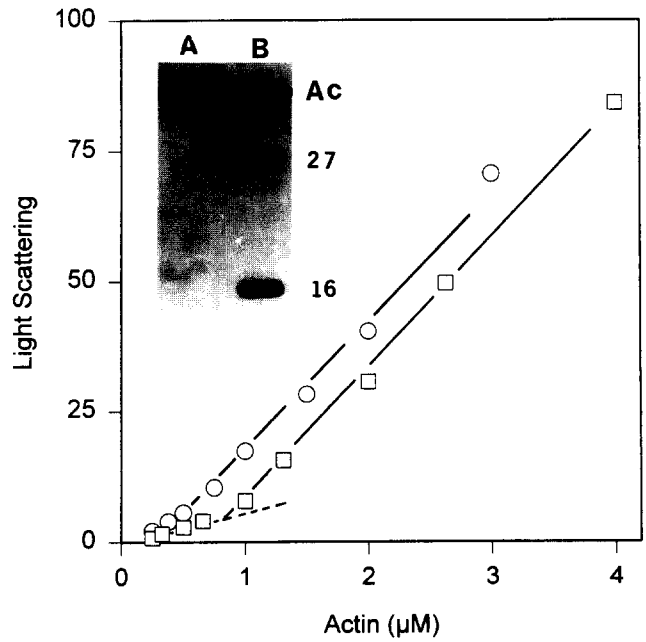

Fig. 2. Critical concentration for polymerization of actin. The extent of actin polymerization by $MgCl_2$ (2.0 mM) was measured by light scattering at 325 nm. Critical concentrations for intact (○) and 27/16 kDa cleaved (□) actin were determined by extrapolating the curves to zero scattering intensity or from the intersection with the G-actin scattering curve (dashed line). Inset, SDS-PAGE of intact and 85% cleaved (27/16 kDa) actin used in these experiments. Lanes A and B in the inset correspond to intact and cleaved actin, respectively. Actin (Ac) and actin fragments (27 and 16 kDa) are identified next to the bands.

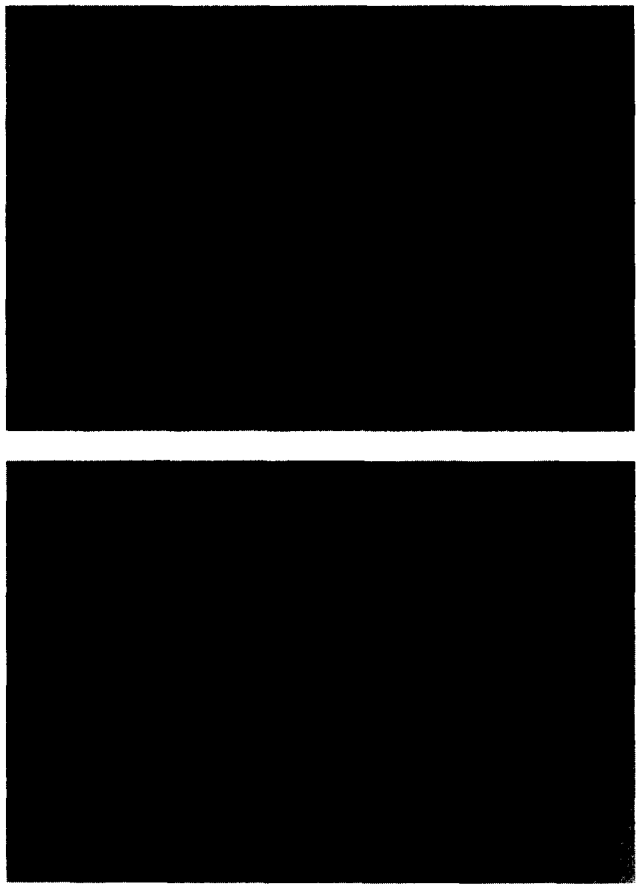

Fig. 3. Electron micrographs of intact (upper panel) and 27/16 kDa cleaved (lower panel) actin. Bar designates length of 0.1 μm.

the rates of cleaved actin polymerization (at 10 μM) and did not change the appearance of actin filaments in electron microscopy observations (Fig. 3). However, while vortexing of actin prior to grid preparation did not alter the size of intact filaments, this procedure fragmented the cleaved actin filaments (data not shown). This indication of lower structural stability of cleaved actin filaments was confirmed by CD melting experiments. In agreement with a recent calorimetric study (10), the main unfolding transition of F(MgADP·BeF_x)-actin, as reported by $[\Theta]_{222}$ elipticity changes, was centered at 78.5 ± 1.0°C. After the cleavage this transition was shifted to 68.1 ± 1.0°C revealing a lower thermostability of the modified protein.

3.3. *Functional properties of the 27/16 kDa actin*

The acto-S1 ATPase activities of intact and the 27/16 kDa cleaved actin, both complexed with BeF_x, were determined after removing the free BeF_x from protein solutions [6]. Michaelis–Menten analysis of the actin concentration dependence (between 0 and 50 μM actin) of acto-S1 ATPase activities showed a considerable decrease in V_{max} value, from 18.2 ± 0.6 to 15.0 ± 0.4 s^{-1}, upon 27/16 kDa cleavage, while the change in K_m was insignificant (Table 1). In vitro motility of actin filaments was slowed by the 27/16 kDa cleavage in proportion to the decrease in V_{max}, by about 20% (Table 1). Although slower, the cleaved F-actin moved as smoothly in these assays as the intact protein but fragmented more readily during the motion.

The binding of tropomyosin to actin was not affected by the 27/16 kDa cleavage (Table 1). As determined in sedimentation assays 0.15 ± 0.01 and 0.16 ± 0.02 Tm per actin were bound to intact and cleaved actin, respectively, in 1:5 molar ratio mixtures of these proteins (Table 1).

It is important to note that while actin-actin and actin-tropomyosin interactions were unchanged by actin cleavage between Ser^{234} and Ser^{235}, the motile function of actin was inhibited by about 20%. This is particularly interesting since the subdomain 4 cleavage site is not known to be in the vicinity of a myosin binding site, and the cleavage has not changed the K_m value but only the V_{max} of acto-S1 ATPase. The present result is also different from the effect of a specific subtilisin cleavage in subdomain 2 of actin (between Met^{47} and Gly^{48}) on its interactions with myosin. The subdomain 2 cleavage greatly decreased the in vitro motility of actin filaments and increased almost sevenfold the K_m values, but did not change the V_{max} of actomyosin ATPase [11]. While these changes could be ascribed to a weaker binding of myosin·ATP to subdomain 2 cleaved actin filaments, it remains to be determined by what mechanisms the 27/16 kDa cleavage impairs the catalytic and motile functions of actin. This, and the fact that the novel cleavage site on actin opens the subdomain 4 to closer structural and functional scrutiny should make the 27/16 kDa cleaved actin a valuable experimental material.

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