Conformational changes in subdomain-2 of G-actin upon polymerization into F-actin and upon binding myosin subfragment-1

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The susceptibility of subdomain-2 of actin to different proteases has been examined, for G-actin, F-actin, G-actin-S(A), and F-actin-S(A) complexes on a comparative basis. The sites of subtilisin, α-chymotrypsin and trypsin attack, exposed on G-actin, are protected in F-actin, F-actin-S(A) as well as in the G-actin-S(A) complex. In contrast, a new cleavage site (Arg39-His40) for ArgC protease, which is protected in G-actin, is exposed in G-actin-S(A) as well as in F-actin and F-actin-S(A). These results are consistent with the previously proposed structural analogy between the ternary (G-actin)S complex and the F-actin-S complex, and provide information on the mechanism of S-induced polymerization of G-actin.

Actin; Myosin; Actomyosin interaction; Conformational change; Limited proteolysis

1. INTRODUCTION

It is well known that myosin subfragment-1 (S,) induces the polymerization of G-actin into decorated F-actin filaments, even in low ionic strength buffers in which actin alone remains monomeric [1,2]. We have shown [3] that the first intermediate in the assembly pathway was a ternary G,S complex, in which S, interacts tightly with 2 G-actin molecules, in low ionic strength buffer and in the absence of ATP. The G,S complex then self-assembles into short (G& oligomers [4]. EDC-crosslinking experiments [5] have shown that the electrostatic close contacts between actin and myosin were very similar, if not strictly identical, in G,S and F-acto-S complexes. These data suggested that the orientation of the two actin molecules interacting with S, in the G,S complex, might be structurally similar to the F-acto-S complex, in which S, interacts mainly with the N-terminal and C-terminal regions in subdomain-1 of one actin subunit, and more weakly with the protruding helix 79-95 of a second actin subunit, adjacent to the first one at the barbed end, along the long pitch helix of the filament [6,7]. In this longitudinal actin dimer-S complex, subdomain-2 of the second actin molecule appears 'clamped' by the myosin head. Conformational changes of actin subdomain-2 therefore are expected to occur upon formation of F-actin, or G,S or F-acto-S complexes. In the present work, the nature of such structural changes has been investigated by limited proteolysis, since this region of actin is known to be susceptible to proteolytic attack [8]. The results show that the Arg39-His40 bond, which is protected in the G-actin state, is exposed to ArgC cleavage both in G-actin-S, F-actin and F-actin-S, complexes; on the other hand, loop 45-52 and segment 61-69 which are susceptible to subtilisin, α-chymotrypsin and trypsin on G-actin, are both protected from proteolytic attack in the G-actin-S, F-actin and F-actin-S complexes. Our results complement and extend the recent work of Chen et al. [9], and are in support of the previously proposed structure of the G,S complex [3-5], and of its being a precursor in S-induced polymerization of G-actin.

2. MATERIALS AND METHODS

G-actin and myosin were purified from rabbit skeletal muscle as described [3-5]. G-actin (~2 mg/ml) was stored on ice in buffer G (5 mM Tris-HCl pH 7.8, 0.1 mM CaCl2, 0.2 mM DTT, 0.2 mM ATP, 0.01% NaN3) and used within 2 weeks. The S(A,) and S(A,) isoforms of chymotryptic myosin subfragment-1 were resolved by cation exchange chromatography on SP-trisacryl (IBF) [5]. Trypsin (TPCK-treated) and α-chymotrypsin were purchased from Worthington. Arg-C protease was from Boehringer and subtilisin carlsberg (P5380) from Sigma.

Before each experiment actin and S,(A,) were dialyzed separately overnight against buffer G0, which is buffer G without ATP. The G-actin-ATP 1:1 complex was then prepared by Dowex-1 treatment of the G-actin solution [3].

All proteolytic digestions of G-actin, S, and G-actin-S, complexes were done with 5 μM G-actin and 5 μM S,(A,) at 10°C in buffer G0 (or 5 mM HEPES, pH 8.0, 0.1 mM CaCl2 in the case of ArgC digestion). Under these conditions no polymerization into decorated filaments took place in the time course of the experiments [5], which was further controlled by light scattering. Proteolytic digestion of F-actin and F-actin-S(A) complexes were done under identical conditions except for the presence of 0.1 M KCl in the buffer. F-actin had been prepolymerized for 90 min at 22°C in the presence of 0.1 M KCL. The digestions were done under the following conditions: trypsin 5 μg/ml, 5-30 min. digestion was stopped by addition of 10 μg/ml trypsin.
soybean inhibitor; a-chymotrypsin 0.1 mg/ml, 3 min, digestion was stopped by 1 mM PMSF; subtilisin 0.1 µg/ml, 5-30 min, reaction was stopped by 1 mM PMSF; ArgC 20 U/ml, 30 min, digestion was stopped by rapid thermodenaturation at 100°C following addition of boiling 5 mM β-mercaptoethanol and 2% SDS.

Samples were denatured and submitted to SDS-PAGE according to Laemmli [10] using a 8% to 18% acrylamide gradient and a Tris-borate electrophoresis buffer [8]. 20 µg of actin and/or 50 µg S,(A,) were loaded in the slots. Molecular weight markers were: myosin (212 kDa), α2 macroglobulin (170 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), transferrin (76 kDa), bovine serum albumin (67 kDa), glutamic dehydrogenase (53 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (soybean) (21 kDa), α-lactalbumin (14 kDa).

The location of ArgC cleavage site on G-actin was determined by sequencing the 37 kDa proteolytic fragment, following SDS-PAGE and electrotransfer on a PVDF membrane (Problot) using an Applied Biosystem instrument and 50 mM Tris-100 mM borate pH 8.0 buffer. Sequencing was performed in gas phase by sequential Edman degradation.

3. RESULTS

The regions of the actin molecule that are susceptible to proteases are known to be located essentially in subdomain 2 [8]. The cleavage sites of subtilisin (Met4′-Gly40), α-chymotrypsin (Met4′-Val45 and Leu67-Lys68) and trypsin (Arg62-Gly63 and Lys68-Tyr69) have been well characterized [8,11-13]. The susceptibility to these proteases as well as to ArgC proteolysis was examined for G-actin, F-actin, G-actin-S,(A,) and F-actin-S,(A,) complexes on a comparative basis. Fig. 1 shows that subtilisin attack was more effective on G-actin (lane c) than on F-actin (lane d), and that binding of S,(A,) exerted a protective effect, both in G-actin-S,(A,) (lane f) and F-actin-S,(A,) (lane g) complexes. S,(A,) was practically not cleaved by subtilisin under the same conditions (lane e). This result is at variance with the recent report by Chen et al. [9] who found that binding of S,(A,) to G-actin did not protect G-actin against subtilisin attack; the most likely explanation for this discrepancy is the presence of 0.2 mM ATP in the digestion assay used in [9], which is known to weaken the G-actin-S,(A,) interaction [3].

The results concerning α-chymotrypsin digestion of actin are displayed in Fig. 2. The data show that both cleavages of G-actin at Met4′ and Leu67, leading to the 35 kDa and 33 kDa fragments (lane c) are greatly inhibited when S,(A,) is bound to G-actin (lane f): only the 35 kDa (30% of the total actin) and no 33 kDa polypeptides were observed under conditions where G-actin alone was 90% proteolyzed into 33 kDa. When F-actin was submitted to α-chymotrypsin attack, only a low amount of 35 kDa was produced, and no 33 kDa (lane d). Total protection of the first cleavage was afforded by S,(A,) binding to F-actin (lane g). One also should
Fig. 2. \( \alpha \)-Chymotrypsin digestion of G- and F-actin in the presence and absence of \( S_i(A_2) \). Actin and/or \( S_i(A_2) \) were submitted to limited \( \alpha \)-chymotryptic digestion as described in section 2. Experimental conditions are as under Figure 1. Lane a, undigested ATP-G-actin (1:1 complex); lane b, undigested \( S_i(A_2) \); lane c, ATP-G-actin + \( \alpha \)-chymotrypsin; lane d, F-actin + \( \alpha \)-chymotrypsin; lane e, \( S_i(A_2) + \alpha \)-chymotrypsin; lane f, G-actin-S\( \gamma \)-A\( \gamma \) + \( \alpha \)-chymotrypsin; lane g, F-actin-S\( \gamma \)-A\( \gamma \) + \( \alpha \)-chymotrypsin; lane h, molecular weight markers.

Note that the \( \alpha \)-chymotryptic cleavage of \( S_i(A_2) \) heavy chain was protected by G-actin, and to a greater extent F-actin binding (compare lanes b, e, f, g). Again our results agree partially with Chen et al. [9] who observed essentially no protection of \( S_i(A_2) \) against \( \alpha \)-chymotryptic cleavage of G-actin at Met\( ^{44} \).

The results of trypsin digestion are shown in Fig. 3. In agreement with Chen et al. [9], only a 34 kDa fragment (corresponding to the second trypsin cleavage site at Lys\( ^{68} \)) was obtained when G-actin was treated by trypsin (lane d), under all conditions of protease concentration and digestion times. Complete protection of the trypsin cleavage was observed in F-actin (lane e), as well as in G-actin-S\( \gamma \)-A\( \gamma \) (lane g) and F-actin-S\( \gamma \)-A\( \gamma \) (lane h) complexes. Note that, as observed by Chen et al., the protective effect of \( S_i \) was exerted despite its extensive cleavage into 75 kDa, 50 kDa and 20 kDa fragments during the digestion.

The results of ArgC digestion shown in Fig. 4, demonstrate that while G-actin was not cleaved by ArgC (lane c), a fragment of 37 kDa was obtained when F-actin was digested by ArgC (lane d). The cleavage was also observed in G-actin-S\( \gamma \)-A\( \gamma \) complex (lane g), and somewhat more accentuated in F-actin-S\( \gamma \)-A\( \gamma \) complex (lane e). In agreement with a previous report [14], binding of actin (both G-actin and F-actin) to \( S_i \) protected against ArgC cleavage of \( S_i \) at Lys\( ^{68} \). When ArgC proteolysis was carried out using G-actin fluorescently labeled on Cys\( ^{174} \) by DACM [14], the 37 kDa fragment was fluorescent (data not shown), showing that it was a C-terminal fragment. Sequencing of the 37 kDa polypeptide yielded HQGVM only as N-terminal sequence, consistent with the location of ArgC cleavage at the Arg\( ^{39} \)-His\( ^{40} \) bond. Therefore this bond is more exposed in the actin-S\( \gamma \) complexes and in the F-actin state than in the G-actin state. It is noteworthy that in the presence of EDTA, which leads to actin denaturation, via the sequential dissociations of tightly bound Ca and ATP [16], a similar proteolytic cleavage of the Arg\( ^{39} \)-His\( ^{40} \) bond was obtained by thrombin [8].
Fig. 3. Tryptic digestion of G- and F-actin in the presence and absence of S$_i$(A$_3$). Actin and/or S$_i$(A$_3$) were submitted to limited trypsin digestion for 8 min as described in section 2. Experimental conditions are as in Fig. 1. Lane a, molecular weight markers; lane b, undigested actin; lane c, undigested S$_i$(A$_3$); lane d, ATP-G-actin (1:1 complex) + trypsin; lane e, F-actin + trypsin; lane f, S$_i$(A$_3$) + trypsin; lane g, G-actin-S$_i$(A$_3$) + trypsin; lane h, F-actin-S$_i$(A$_3$) + trypsin.

4. DISCUSSION

Proteolytic digestion by subtilisin, α-chymotrypsin, trypsin and ArgC has been used to examine the conformation changes of regions of subdomain-2 of actin upon filament assembly, and upon interaction of G-actin and F-actin with the myosin head. Two main observations have been made: upon binding of S$_i$ to G-actin, the segments 44–50 and 60–69 are protected from proteolytic attack, while the 39-40 bond is exposed to ArgC proteolysis. Similar changes in subdomain 2 occur upon polymerization of G-actin in filaments. Therefore these regions of subdomain-2 of actin adopt, in the G$_i$S complex, a conformation very similar to the conformation in the F-actin state. While binding of S$_i$ to G-actin greatly affects the conformation of subdomain-2, binding of S$_i$ to F-actin does not greatly affect its susceptibility to proteases.

These results do not prove, but are supportive of the structure proposed [3–5] for the G$_i$S complex, within which the two G-actin molecules interacting with the myosin head are connected to each other via longitudinal bonds similar to the actin-actin bonds along the long pitch helix of the F-actin filament. In this interaction, subdomain-2 of the pointed end of one actin molecule interacts with subdomain-1 at the barbed end of the other molecule. This interaction would be responsible for the change in subdomain-2 leading to the exposure of the Arg$^{39}$-His$^{40}$ bond and protection of segments 45–52 and 60–69. These conclusions agree with the structural differences in subdomain-2 between F- and G-actin that could be recently estimated from a Monte Carlo refinement of the positions of the actin domains [17].

REFERENCES

Fig. 4. ArgC digestion of G- and F-actin in the presence and absence of $S_1(A_2)$. Actin (3 μM) and/or $S_1(A_2)$ (3 μM) were submitted to ArgC digestion as described in section 2. Lane a, molecular weight markers; lane b, undigested actin and $S_1(A_2)$; lane c, ATP-G-actin (1:1 complex) + ArgC; lane d, F-actin + ArgC; lane e, F-actin-$S_1(A_2)$ + ArgC; lane f, $S_1(A_2)$ + ArgC; lane g, G-actin-$S_1(A_2)$ + ArgC.