A Ca^{2+}-dependent actin modulator from vertebrate smooth muscle

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A protein of M_r ~85000 has been isolated and purified from pig stomach smooth muscle that modulates the polymer state of actin in a Ca^{2+}-dependent manner. When added either to preformed F-actin filaments or to G-actin, prior to polymerisation, the modulator induces the formation of shorter filaments. The average filament length in the presence of the modulator is directly dependent on its molar ratio to actin indicating a stoichiometric rather than a catalytic type of interaction. When mixed with G-actin the modulator forms a stable complex with two actin monomers; this complex is presumed to act as a potent nucleus for actin polymerisation. The dynamics of the interaction with F-actin suggests a direct severing of actin filaments by the modulator via a binding to intrafilamentous actins.

Smooth muscle *Actin filament* *Actin modulator* *Ca^{2+}-dependence*

1. INTRODUCTION

Among the various proteins that bind to actin one group has been isolated from non-muscle sources that acts specifically on the polymer state of actin in a Ca^{2+}-sensitive manner. Proteins of this group include gelsolin from macrophages [18–20], villin from the microvilli of intestinal brush border [1,4,5], brevin from blood serum [6,7], fragmin from Physarum [8–11] and severin from *Dictyostelium* [2,17]. Because these proteins directly modify the filamentous state of actin the term 'actin modulator' for such proteins has been suggested [10]. Actin modulators of this kind bind to either G- or F-actin and their interaction with actin leads to the formation of shorter filaments apparently by the capping of one filament end and/or via direct fragmentation of filaments.

Although no conclusive evidence exists on the physiological role of these proteins it is assumed that they may have some function in the dynamic transformations of actin from polymer to non-polymer state and vice versa which are known to occur in many non-muscle cells. We describe here for the first time the isolation of a Ca^{2+}-dependent actin modulator from a muscle tissue – vertebrate smooth muscle – for which the polymer state of actin is supposed to be comparatively stable.

2. MATERIALS AND METHODS

The actin modulator from pig stomach smooth muscle (PSAM, pig stomach actin modulator) was purified by a modification of the method described for fragmin from *Physarum* [10]. Muscle tissue prepared from pig stomachs was minced and homogenized [16] with 5 vols of extraction medium (20 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 1 mM DTE, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 mM imidazole, pH 7.0). After centrifugation, the extract was fractionated with ammonium sulfate and the 40–55% pellet subjected to the following purification steps: (1) ion exchange chromatography on DEAE-Sepharose CL-6B with a 0.05–0.35 M KCl gradient for elution; (2) gel filtration on Ultrogel AcA 34; (3) hydroxyapatite chromatography, eluting with a PO_4-gradient from 0.05–0.3 M; and (4) a second ion exchange chromatography in the presence of 6 M urea to separate the complex between actin and the modulator. After dialysis against 10 mM imidazole, 1 mM DTE and 0.5 mM EGTA the

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modulator was completely renatured. EGTA (1 mM) was included in all solutions during purification except during hydroxyapatite chromatography. The modulating activity of the various fractions was assayed by viscometry as in [9,10]. Comprehensive details of the purification procedure will be given elsewhere (in preparation). All experiments described here were performed using skeletal muscle actin purified as in [17] with a final gel filtration on Sephadex G-150. Viscosity measurements were made with an Ostwald type capillary viscometer with 15 s outflow time for water at 25°C. Protein concentration was determined by the biuret method. SDS-gel electrophoresis was performed on gels containing an 8–18% gradient of polyacrylamide using the buffer system in [14]. Negative staining was done on carbon films using 1% aqueous uranyl acetate.

3. RESULTS

3.1. *Purification of PSAM*

By the method indicated we obtained between 20–25 mg purified protein from 1 kg muscle tissue. The protein appeared homogeneous according to SDS-electrophoresis (fig.1c) and exhibited a single band of $M_r \sim 85\,000$ in our electrophoresis system. Gel filtration experiments revealed that the protein forms no aggregates and elutes as one component with a Stokes radius of 3.8 nm corresponding to an M_r at 78 000 for a globular protein (see also fig.5). The most effective purification was achieved in the first DEAE chromatography where the activity was eluted as one sharp peak in which the modulator was present mainly complexed with actin. A complete separation of actin from the modulator was only possible after the second DEAE chromatography in the presence of urea.

3.2. *Effect of PSAM on the viscosity of actin*

PSAM has a marked influence on the viscosity of actin either when added to G-actin before or even synchronously with the salt to induce polymerization or to already assembled F-actin at steady state. As shown in fig.2 PSAM markedly enhanced the initial rate of viscosity increase as compared to the control, indicating a strong nucleating effect on actin polymerization, and reduced the steady-state viscosity after polymerization. Both effects were increased with increasing

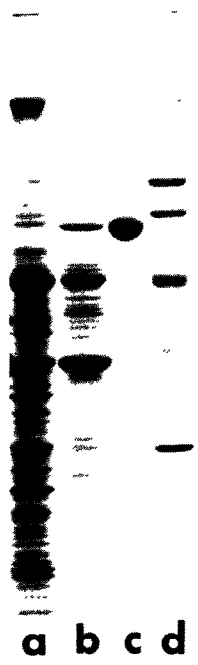


Fig. 1. SDS-gel electrophoresis of fractions from various purification steps of PSAM. (a) Extract; (b) pooled fractions after the first DEAE column; (c) purified PSAM; (d) M_r standards: myosin heavy chain 205 000, β-galactosidase 116 000 phosphorylase *b* 94 000, bovine serum albumin 67 000, ovalbumin 45 000, carbonic anhydrase 29 000.

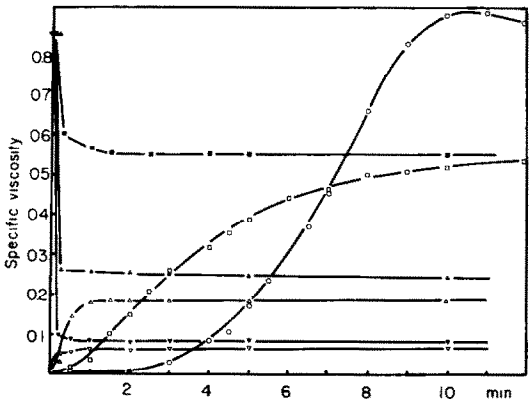


Fig. 2. Influence of PSAM on the viscosity of actin. Skeletal muscle G-actin (1 mg/ml in 1 mM ATP, 0.2 $CaCl_2$ and 10 mM imidazole, pH 7.4) was polymerized by the addition of 70 mM KCl. PSAM at various molar ratios [PSAM:actin = 1:30 (▼,▽), 1:100 (▲,△), 1:300 (■,□)] was added either before polymerization (open symbols) or to F-actin at steady-state (closed symbols). (○) Actin control.

amounts of modulator. In addition, at low ratios of modulator to actin a significant delay in approach to steady-state viscosity was observed despite the increase of the initial rate via nucleation (fig.2). At higher amounts of modulator the rate of initial viscosity increase became so marked that it overcame the delay phase. When the free Ca^{2+} concentration was below 10^{-6} M spontaneous nucleation was increased per se but practically no difference was observed in the presence or absence of the modulator indicating no or very little interaction between modulator and actin at low Ca^{2+}-concentrations (not shown).

The addition of PSAM to F-actin led to a rapid drop of viscosity. For all concentrations of modulator used, the reaction was almost complete within less than 30 s with only a small additional decrease of viscosity thereafter. Again this effect was completely Ca^{2+}-dependent.

The change in the steady-state viscosity of actin produced by PSAM may be taken as a measure of its activity. As shown in fig.3, the relationship of steady-state viscosity and different PSAM: actin ratios (G- or F-actin) fits to a hyperbolic curve. The effect on F-actin was somewhat less pronounced than on G-actin. A 50% reduction of steady-state viscosity was obtained at molar ratios of 1:550 and 1:400, respectively. At high molar ratios the viscosity approached that of G-actin.

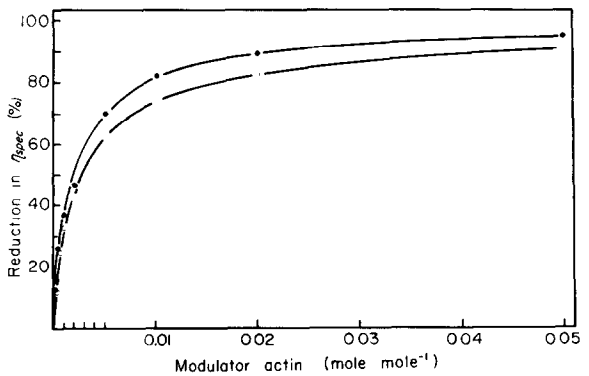


Fig. 3. Reduction in steady-state viscosity of actin as a function of the molar ratio of PSAM and actin. (●) PSAM added to actin before polymerization, (○) PSAM added to F-actin. Conditions: 1 mg/ml actin, 1 mM ATP, 0.2 mM $CaCl_2$, 10 mM imidazole, pH 7.4. KCl (100 mM) and $MgCl_2$ (2 mM) were added for polymerization. Viscosity was measured 10 min after addition of salt or PSAM, respectively.

Table 1

Average filament length after addition of PSAM to F-actin

Actin:PSAM (M/M)	Average filament length (nm)	Actin molecules per average filament
100:1	241	87
50:1	125	45
20:1	66	24
10:1	39	14

Electron micrographs taken from the various samples shown in fig. 4 were enlarged to 125 000 × and the lengths of ~500 filaments for each sample measured with a modified map tracer. From the number average filament lengths the number of actins per average filament was calculated on the basis of 13 actin molecules per 36 nm filament length.

3.3. *Influence of PSAM on actin filament length*

Electron microscopy of actin filaments incubated with various amounts of PSAM revealed a significant filament shortening at low molar ratios (fig.4b,c). At higher ratios only short fragments or even smaller non-filamentous aggregates were present (fig.4d,e). The distribution of filament lengths was heterogeneous but measurements from electron micrographs of the number average length of filaments revealed a close proportionality with the molar ratio of actin to modulator (table 1). Comparison of a given molar ratio with the number of actin molecules calculated for the average filament, indicated that there should be only one molecule of modulator per filament. At high modulator:actin ratios ($\geqslant$1:10) the proportionality no longer held owing to the formation of increasing numbers of small, non-filamentous aggregates.

3.4. *Formation of a 2:1 complex of actin and PSAM*

The interaction of PSAM and actin was also shown by gel chromatography (fig.5). As mentioned above the modulator eluted as a single peak and formed no aggregates. If a 2:1 molar mixture of actin:PSAM was applied to a column again a single peak was eluted but with an apparent M_r of 160 000 indicating a stable 2:1 complex of actin and PSAM. For a 1:1 molar mixture of both pro-

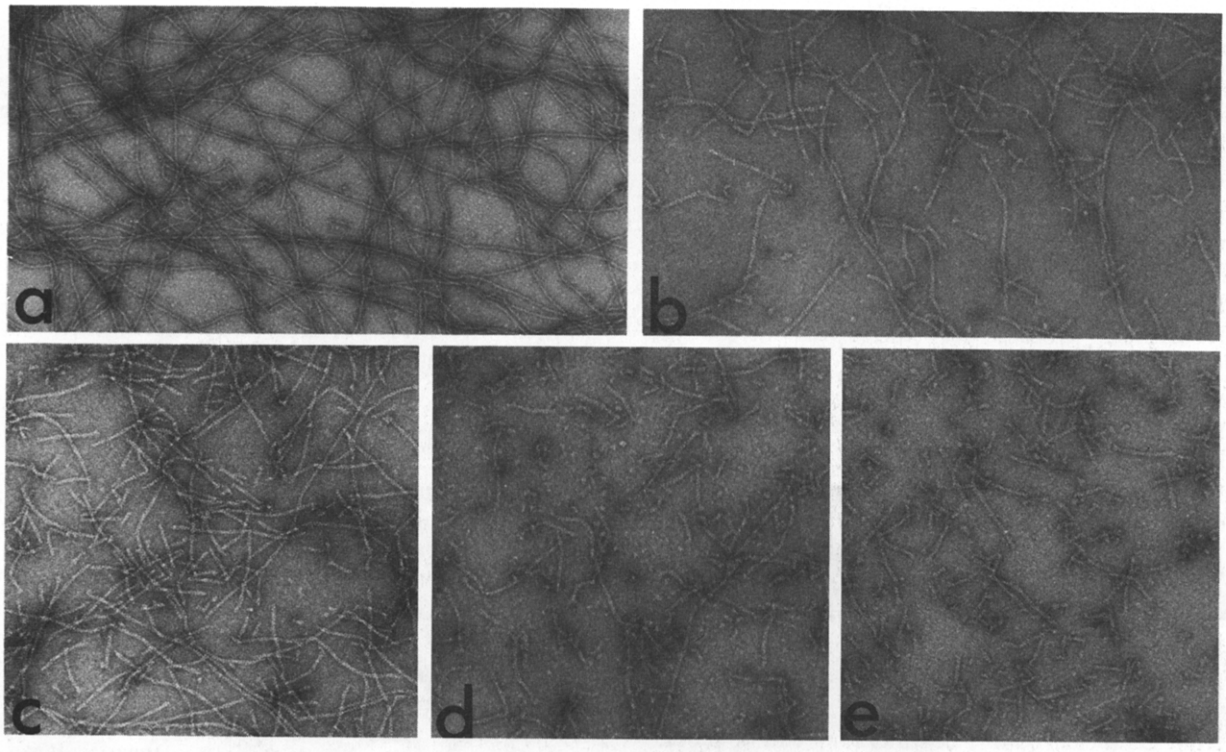
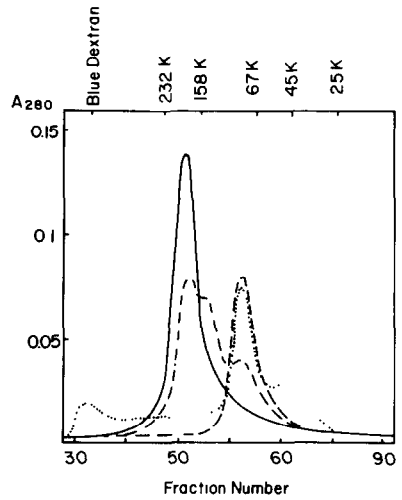


Fig. 4. Electron micrographs of actin filaments after addition of PSAM. (a) F-Actin without PSAM; (b–e). F-actin to which PSAM had been added at a molar ratio of 1:100 (b), 1:50 (c), 1:20 (d) and 1:10 (e). (a) 50 000 ×, (b–e) 75 000 ×.



teins a heterogeneous elution profile of 3 components was obtained corresponding to the modulator alone, the 2:1 complex and an intermediate component, presumably a 1:1 complex of both proteins. This situation may be explained by the modulator having two binding sites for actin with approximately the same affinity. At higher molar ratios of actin:PSAM the distribution became again heterogeneous with part of the actin eluting in the void volume and part in the position corresponding to G-actin. In the presence of EGTA no complex formation was observed.

Fig. 5. Gel filtration of PSAM and actin–PSAM mixtures. Samples (1 ml) were applied to a 110 × 1.5 cm column of Ultrogel AcA 34 and eluted at a flow rate of 9 ml/h with 0.1 M KCl, 0.5 mM DTE, 10 mM imidazole (pH 7.2), containing 0.2 mM $CaCl_2$ or 1 mM EGTA, respectively. (– – –) PSAM alone, (- - - -) PSAM + G-actin at a molar ratio of 1:1, or (——) 1:2, mixed in a buffer containing 10 mM imidazole, 0.5 mM DTE and 0.2 mM $CaCl_2$; (· · ·) PSAM + G-actin mixed at a molar ratio of 1:2 in the same buffer containing 1 mM EGTA instead of $CaCl_2$. The reference scale at the top margin was obtained with a set of standard proteins containing catalase, aldolase, BSA, ovalbumin and chymotryosinogen A.

4. DISCUSSION

The abundance of actin binding proteins in non-muscle cells that show specific effects on the polymer state of actin (reviews [3,18]) apparently reflects the functional requirements for the polymerization and depolymerization of actin in normal cell motility and locomotion.

Such dynamic reactions are not assumed to be involved in the function of muscle cells and the only muscle protein yet known to affect the polymerization of actin is β-actinin from skeletal muscle [15]. Although β-actinin also promotes nucleation of actin polymerization it differs substantially from the modulator described here, namely by the lack of Ca^{2+}-dependence and its inability to fragment F-actin. β-Actinin would seem to belong to a class of actin capping proteins, found in non-muscle cells [12,13], which exert their effect exclusively by binding to one of the ends of the actin filament; β-actinin apparently binds to the slow polymerizing end.

We shall show elsewhere that the PSAM described here blocks the fast polymerizing end of the actin filament. This is consistent with the observation that, under suitable conditions, the polymerization during the elongation phase of the filaments was delayed. However, the effects of the modulator on actin cannot be explained simply by a capping process since its reaction with F-actin is too fast to be due to a mere reorganization of actin filaments after capping. High-resolution time course measurements of this reaction down to 2-s intervals have established that the shortening of filaments occurs within ~6 s (in preparation). This can be explained only by a direct severing action of the modulator via a binding alongside the filament to intrafilamentous actins. The protein from non-muscle sources most closely related to the modulator seems to be gelsolin which shows a Ca^{2+}-dependence, acts on F-actin, binds 2 actin monomers [21] and, unlike villin [1], lacks actin bundling activity in the absence of Ca^{2+}. This analogy is supported by the immunological cross-reaction of anti-gelsolin with uterine smooth muscle tissue [23].

The pattern of phenomena resulting from the interaction of PSAM and actin is complex: at stoichiometric ratios to actin it can either completely depolymerize actin or inhibit polymerization by formation of a stable 2:1 complex with actin which is unpolymerizable. In substoichiometric amounts with either G- or F-actin it induces the formation of short filaments whose length is dependent on the molar ratio of the 2 proteins. It influences the time course of actin polymerization by promoting nucleation as well as by decreasing the elongation rate, and its activity can be completely regulated by Ca^{2+} concentrations in the physiological range. It remains to be established which of these effects are relevant to its physiological role in the cell.

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