A novel 40000 Da Ca^{2+}-dependent actin modulator from bovine brain

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A monomeric protein of M_r 40000 that modulates the polymer state of actin has been isolated from bovine brain. When added either to preformed actin filaments or to monomeric actin, prior to polymerization, the modulator reduces the low-shear viscosity of F-actin provided that Ca^{2+} is present. The 40 kDa protein also inhibits the rate of actin polymerization. The inhibition is fully suppressed by removal of Ca^{2+} and restored by subsequent readdition of Ca^{2+}, suggesting that the Ca^{2+}-controlled interaction of actin with the 40 kDa modulator is freely reversible.

(Brain) Actin filament Actin modulator Ca^{2+} dependence

1. INTRODUCTION

Actin is supposed to play a role in various neuronal activities including release of neurotransmitters [1], axonal transport [1–3], and 'twitching' of dendritic spines [4]. These dynamic events require both a spatial and temporal control of the diverse forms of actin. The actual state of actin at any particular place and time within neurones seems to result from a very delicate interplay of accessory proteins which modify actin polymerization and actin filament structure [5]. Therefore, the knowledge of potential actin-associated proteins in the nervous tissue is of importance for understanding the actin-involving events. Among several actin-binding proteins that have been isolated from brain [6–15], two were reported to be Ca^{2+}-sensitive: a 105 kDa α-actinin-like protein that cross-links actin filaments [14] and a 90 kDa gelsolin-like protein [7] that caps [13] and fragments [11] actin filaments. We describe here for the first time the isolation of a Ca^{2+}-dependent actin-binding protein of M_r 40000 from bovine brain.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Proteins*

All buffers and protein solutions contained 5 mM 2-mercaptoethanol, 0.6 mM NaN_3, 0.4 mM PMSF and 1.5 μM pepstatin. Actin was isolated from rabbit skeletal muscle according to [16]. The 40 kDa actin-modulating protein from brain (BAM-40, brain actin modulator-40) was prepared from bovine tissue. During preparation, brain fractions with Ca^{2+}-dependent actin-modulating activity were identified by their ability to reduce the low-shear viscosity of F-actin in a Ca^{2+}-dependent manner. Brain tissue was homogenized with 1 vol. of a solution containing 0.1 mM $CaCl_2$ and SAT buffer (0.34 M sucrose, 1 mM ATP, 20 mM Tris-HCl, pH 8.0) and then centrifuged at 28000 × g for 40 min. The pellet was washed once with 1 vol. of the same solution and then extracted with 1 vol. of a solution con-

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taining 3 mM EGTA and SAT buffer. Following centrifugation, the extract was subjected to the batch treatment with DE52 cellulose equilibrated in 0.1 mM $CaCl_2$, 50 mM KCl and 10 mM Tris-HCl pH 7.8. The material not absorbed was fractionated with ammonium sulfate. The 20–60% pellet was dialyzed against 0.1 mM $CaCl_2$, 10 mM sodium citrate, pH 6.0, and applied to a CM52 cellulose column equilibrated in the same solution. The active fractions were eluted with a linear KCl gradient (0–0.1 M), then pooled and concentrated by Amicon ultrafiltration with a YM5 membrane followed by chromatography on a Sephacryl S200 column equilibrated in 0.1 mM $CaCl_2$, 100 mM KCl and 20 mM imidazole, pH 7.2. The pure BAM-40 was obtained by a final ion-exchange chromatography on DE52-cellular equilibrated in 0.1 mM EGTA and 10 mM Tris-HCl, pH 7.8, and eluted with a linear KCl gradient (0–0.1 M).

2.2. *Viscometry*

Viscosity was measured at low-shear rates at 27°C using the falling ball viscometer (100 μl capillary) of [17]. Kinematic viscosity was measured at high-shear rates at 27°C in an Ostwald type capillary viscometer (275 μl) with 64 s outflow time for water.

2.3. *Protein and calcium analysis*

Protein concentrations were determined as in [18]. SDS-PAGE was performed on slab gels containing 12.5% of acrylamide according to [19]. The gels were stained as in [20]. The free [Ca^{2+}] was controlled using an EGTA buffer system as in [21].

3. RESULTS

3.1. *Purification of BAM-40*

During the last step of purification (DE52-cellulose chromatography), the activity which reduced the low-shear viscosity of F-actin in a Ca^{2+}-dependent manner was eluted at 15 mM KCl and coincided with a single polypeptide of M_r 40000, as found by SDS-PAGE (fig.1). When applied to a calibrated column of Sephacryl S200, the protein was eluted as one component with an M_r of about 40000 for a globular protein. These results indicate that BAM-40 is a monomeric protein.

Fig.1. SDS-PAGE of purified BAM-40 (a) and actin (b). The M_r values ($\times 10^{-3}$) of standard proteins are indicated.

3.2. *Effect of BAM-40 on the low-shear viscosity of actin filaments*

BAM-40 markedly reduced the low-shear viscosity of F-actin when added either to preformed F-actin at steady state or to G-actin before or synchronously with the salt to induce polymerization. As shown in fig.2, the effect was detected at substoichiometric BAM-40 levels provided that Ca^{2+} was present. For preformed F-actin and actin polymerized in the presence of BAM-40, a 50% decrease in viscosity was attained respectively with 25 and 8 nM BAM-40, corresponding to BAM-40 to actin molar ratios of about 1:300 and 1:1000. In the absence of Ca^{2+} (0.4 mM EGTA), BAM-40 was completely inactive. Inset in fig.2 shows the range of free [Ca^{2+}] required for BAM-40 action. The maximal reduction of actin viscosity occurred at free [Ca^{2+}] around 30 nM which is within the limits observed in living cells.

3.3. *Effects of BAM-40 on actin polymerization*

Under very favorable conditions for actin polymerization, such as those used for viscosity measurements at low-shear-rates (2 mM $MgCl_2$

with 100 mM KCl), the effect of substoichiometric amounts of BAM-40 on both the rate and extent of spontaneous polymerization was barely detectable, as monitored by high-shear viscometry. In conditions where the polymerization of actin alone was slow, BAM-40 markedly inhibited this process provided that Ca^{2+} was present. In 40 mM KCl, for instance, 50 mM BAM-40 prolonged by a factor of 5 the lag prior to the viscosity increase and reduced by about 80% the maximum rate of the viscosity change (fig.3, c compared to b). Both effects were enhanced with increasing amounts of BAM-40. Under all conditions tested, no significant effect of BAM-40 on the steady-state high-shear viscosity of actin filaments was found. Even in the presence of 100 nM BAM-40, which reduced the low-shear viscosity of actin by more than 95%, the Ostwald viscosity after polymerization was identical within 5% to the one in the absence of BAM-40.

In the absence of Ca^{2+}, spontaneous nucleation of actin alone was increased (fig.3, a compared to b) but no difference in the kinetics of actin polymerization was observed in the absence or

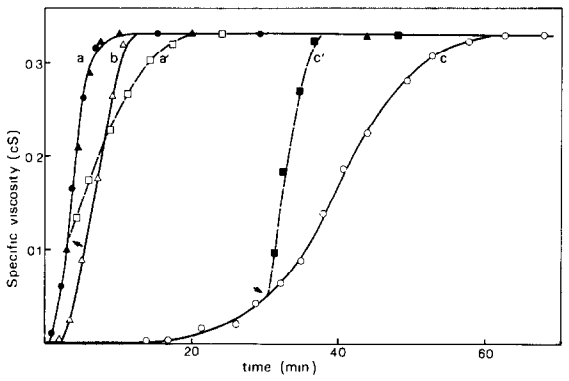

Fig.3. Effect of BAM-40 on the kinetics of actin polymerization monitored by high-shear viscosimetry. Actin monomer (7.6 μM) was mixed with 0 (△, ▲) or 50 nM (○,●) BAM-40 and polymerized in 40 mM KCl, 1 mM ATP, 10 mM Tris-HCl, pH 7.8, and either 0.1 mM $CaCl_2$ (○, △) or 0.4 mM EGTA (●, ▲). At the times indicated by arrows, $CaCl_2$ was brought to 0.5 mM in the sample containing BAM-40 and EGTA (●⟶□) or EGTA (0.5 mM) was added to the sample containing BAM-40 and $CaCl_2$ (○⟶■).

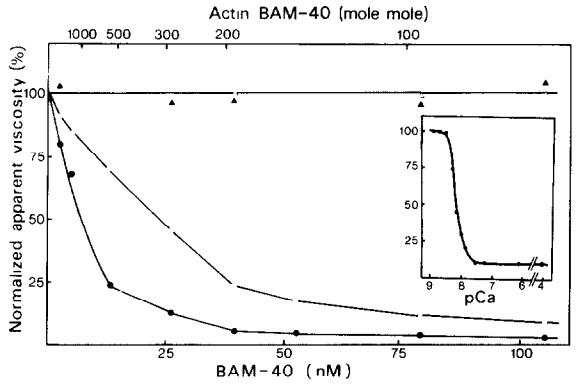

Fig.2. Effect of BAM-40 on the low-shear viscosity of actin filaments. BAM-40 was added to G-actin before polymerization (●, ▲) or to F-actin at steady-state (○, △). Viscosity was measured 30 min after addition of salt (●, ▲) or BAM-40 (○, △), respectively. Conditions: 7.6 μM actin, 2 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 10 mM Tris-HCl, pH 7.8, and either 0.1 mM $CaCl_2$ (○,●) or 0.4 mM EGTA (△, ▲). Inset: Calcium dependency of the reduction in low-shear viscosity of F-actin by BAM-40. p*Ca* is the negative logarithm of the molar concentration of free Ca^{2+}. Viscosity was measured 30 min after synchronous addition of BAM-40 (30 nM) and salt to monomeric actin.

presence of BAM-40 (fig.3a). When the lag phase was terminated in the presence of BAM-40, the addition of Ca^{2+} reduced the rate of the viscosity change to a value similar to that where both Ca^{2+} and BAM-40 were present from the beginning to the end of the polymerization process (fig.3, a′ compared to c). This indicates that the calcium-form of BAM-40 slows actin filament elongation. The effect of BAM-40 on the rate of the viscosity increase was rapidly reversed by removal of Ca^{2+} during the course of slowed actin polymerization (fig.3c′). The latter curve became parallel with that of actin alone (fig.3, c′ compared to a) suggesting release of BAM-40 from actin filaments. Thus, the Ca^{2+}-controlled interaction of BAM-40 with actin seems to be fully reversible.

4. DISCUSSION

We have shown here the existence in bovine brain of a 40 kDa Ca^{2+}-dependent actin modulator. Under suitable conditions for actin polymerization, the modulator prolonged the lag prior to the viscosity increase and slowed the rate of the viscosity change. Both effects could be due

to either or both of two different mechanisms. BAM-40 could inhibit the formation of actin filament nuclei. Alternatively, the modulator could inhibit the addition of actin monomers to the ends of nuclei and filaments, nuclei being normally formed in the presence of BAM-40. The latter mechanism may account not only for the slower rate of the viscosity increase but also for the longer time required for the growing filaments to reach a length detectable by the viscometric assay. The inhibition of elongation is consistent with the observation that, when actin was polymerized in the presence of BAM-40 and in the absence of Ca^{2+}, the addition of this metal during the elongation phase slowed the rate of the viscosity increase.

By viscometry at high-shear rates we found no substantial decrease in the steady-state size of actin polymers at substoichiometric concentrations of BAM-40 up to the molar ratio of 1:75. Although such viscosity measurements are somewhat poorly sensitive to changes in the molecular mass of polymers and the shear degradation of actin filaments makes different length distributions more similar [22], these results indicate that the modulator does not appreciably shorten the filaments. Thus, BAM-40 does not seem to belong to the class of severing proteins which fragment actin filaments very efficiently at very low concentrations (molar ratio 1:500–1:1000). The strong decrease in the low-shear viscosity of F-actin at substoichiometric levels of BAM-40 may reflect slight changes in filament length, too small to be detected by high-shear viscometry, as well as the loss of interactions between filaments which apparently influence the viscosity of actin networks [23]. The pattern of phenomena described here resembles the effects of cytochalasins on actin [24]. These fungal metabolites reduce the rate of elongation at the fast-growing end of actin filaments [25]. Cytochalasins do not behave as nucleating factors, nor do they sever filaments.

The proteins from other sources most closely related to the modulator seem to be two F-actin-capping proteins from *Physarum polycephalum*, called Cap 42(a) and Cap 42(b) [26]. These proteins of M_r 42000 show a Ca^{2+}-dependence, inhibit actin monomer exchange at the fast-growing end of actin filaments and, unlike *Physarum* fragmin, do not sever filaments. This analogy is of interest since the *Physarum* capping proteins are antigenically related to actin. Further work will be required to determine if the modulator shares some features with actin itself.

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