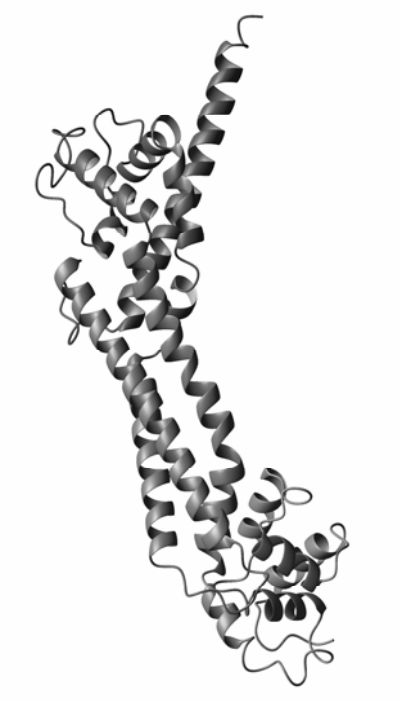
PhD THESIS

The effect of formins on the polymerisation and dynamic properties of actin filaments



BEÁTA BUGYI

University of Pécs
Faculty of Medicine
Department of Biophysics
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The effect of formins on the polymerisation and dynamic properties of actin filaments

Beáta Bugyi

Program: Biochemistry and molecular biology

Head of the program: Dr. Balázs Sümegi

Subprogram: B-130: Investigation of functional protein dynamics with biophysical methods

Head of the subprogram: Dr. Béla Somogyi

Supervisors: Dr. Béla Somogyi, Dr. Miklós Nyitrai

University of Pécs

Faculty of Medicine

Department of Biophysics

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Introduction

The actin cytoskeleton (microfilaments and associated proteins) is one of the three polymer systems, which take part in the construction of protein backbone of the eukaryotic cells. Besides providing the shape and mechanical properties of cells the actin cytoskeleton determine the dynamic behaviour of living cells as well. The structurally different actin filament networks have distinct mechanical properties due to the spatially and temporally well coordinated work of diverse actin-binding proteins.

The main component of the microfilaments is the actin. In cells, actin can be found both in monomeric (globular, G - actin) and filamentous (F - actin) form. The structure of actin monomer can be divided into two domains. Between these domains, the high affinity cation-, and nucleotide binding cleft can be found. The bound divalent cation is Mg^{2+} *in vivo* or Ca^{2+} *in vitro*. The bound nucleotide can be ATP, ADP.P_i or ADP. The assembly of actin monomers into filaments, the polymerization process involves three main phases. After the activation of monomers, in the first step two actin monomers form a dimer. Joining of and additional monomer to actin dimers results in actin trimers, or nuclei. In the elongation phase the actin filaments growth by incorporating the monomers into the filaments. In the last step, in steady – state a so called treadmilling occurs, in which the length of the filaments do not change. In this phase the actin monomers associate to and dissociate from both ends of the filaments with different kinetics. This results in the polarity of actin filaments. At the fast growing end (or barbed end) the association while at the slow growing end (or pointed end) the dissociation of monomers is the dominant process. At each end as the ratio of the dissociation (k_-) and association (k_+) constants of these processes the critical concentration can be defined. As a consequence of treadmilling the value of critical concentration characteristic for steady – state is between the values of the critical concentration of the barbed and pointed end so that its value is closer to the critical concentration of barbed end.

The lifetime of the filaments is determined by the rate of ATP hydrolysis and dissociation of the inorganic phosphate from filaments. The former occurs within seconds (0.3 s^{-1}(1)) after the incorporation of monomers into the filaments, the latter is much slower (0.002 s^{-1}(2)). The phosphate dissociation is accompanied by a conformational change in protomers, which results in the destabilization of actin filaments.

In living cells owing to different extra-, and intracellular stimuli the fast rearrangement of actin cytoskeleton occurs. The dynamic polymerization and depolymerisation of actin filaments are essential in these processes. The rate-limiting step in spontaneous polymerisation of actin is the nucleation phase due to the instability of actin dimers and trimers (3). In cells various mechanisms exist to induce polymerization, which are productively accelerate the production or increase the number of free barbed ends (2).

The nucleation factors (formins, Arp2/3 complex (4,5), spire (6,7)) are actin-binding proteins, which govern the dynamic remodelling of the actin cytoskeletons. Ubiquitous actin nucleation factors are the formin proteins that play essential roles in a wide range of cellular and developmental processes. The defining features of formins are the forming homology domains (FH1, FH2 and FH3 domain (8)). Subsets of formins the Diaphanous-related formins (Dia) involve additional domains, which mediate the regulation of the proteins through intramolecular autoinhibitory interactions (9).

The FH2 domain is the central catalytic element of formins, which responsible for the effect on actin. The FH2 dimer, composed of two antiparallel FH2 domains is the functional form of this domain. The flexible linker region at the N-terminal of the FH2 domain linking it to the FH1 domain is the key structural element in dimerisation (10). The FH2 dimer nucleates actin filament assembly. The FH2 domains from different species partially or completely inhibit filament elongation (11,12). The FH1 domain at the N-terminal of the FH2 domain consists of proline rich sequences providing binding sites for the actin monomer binding protein: profilin (8).

The profilin bound to the FH1 domain promotes the nucleation catalysed by the FH1FH2 domain and increases the rate of elongation compared to the rate of freely elongating barbed ends. The characteristic feature of the FH1FH2 is the processivity: it does not need to dissociate and reassociate to the barbed end every time when a monomer adds or removes but can remain at the barbed end of the filaments as they elongate (13,14).

Despite the numerous studies characterizing the effects of formins on actin the exact mechanism has not been revealed yet. The smallest region of formins, which is essential for their effect on actin polymerisation was not defined. It was also not clarified whether the formins can induce conformational changes within the actin filaments or formins can modify their interactions with other proteins. The three dimensional atomic structure of formins has not been determined.

Aims

The first three dimensional atomic structure, the structure of the core FH2 domain of mDia1 (from mouse) was determined at 2.6 Å resolution within the framework of international collaboration between our laboratory and Professor Alfred Wittinghoffer (Max Planck Institute, Dortmund, Germany). In the present work the core FH2 domains and the FH2 domain involving an additional flexible linker region at its N-terminal from mammalian Dia formins (mDia1, mDia2 and mDia3) were investigated.

Our work was focused on the characterization of the interactions between mDia formins and rabbit skeletal actin *in vitro*. The effects of mDia fragments on the polymerization kinetics and dynamic properties of actin filaments were examined.

- In the first part of our research we described how the mDia fragments modify the polymerization, depolymerisation process and the critical concentration of actin assembly.
- We investigated which residues of the core FH2 domain were important for the effect.
- In the second part of our research we examined how the mDia fragments modify the conformational properties of actin filaments.
- We studied whether the functional properties of actin filaments and their interactions with other proteins were modified in the presence of formins.

Methods

Proteins

Actin from rabbit skeletal muscle was prepared according to the method of Spudich and Watt (15,16)and stored in buffer A (4 mM TRIS - HCl (pH7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT).

The plasmids (PGEX-4-T3) encoding the mDia proteins were obtained from our collaboration partner. The fragments of mammalian formin mDia were expressed as GST-fusion proteins in *E.coli* strain BL21 (DE3) pLysS. The GST-fusion proteins were loaded onto a GSH-column, eluted from the column after cleavage by thrombin and further purified by size exclusion chromatography. After the preparation the formin fragments were kept in –80^{o}C in storing buffer (50 mM TRIS - HCl (pH7.3), 50 mM NaCl and 5 % glycerol).

Cation exchange and polymerisation

After isolation the actin is in monomeric form and binds Ca^{2+}. The bound Ca^{2+} was replaced by Mg^{2+} by adding 200 μM EGTA and 50 μM $MgCl_2$ (17). The Mg^{2+}-actin was polymerized with 10 mM KCl and 0.5 mM $MgCl_2$. The ionic strength dependence of the effect of formins was investigated using 50 mM KCl and 1 mM $MgCl_2$ or 100 mM KCl and 2 mM $MgCl_2$ as well. The ionic strength was calculated from the concentration (c_i) and the charge (z_i) of each ion according of the following equation:

$$\left[ionic\ strength\right] = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \qquad (1)$$

If the sample contained formin fragments that were added to the solution prior to the polymerization. In order to exclude the effect of the storing buffer its volume regardless of whether it was added with or without formins was kept constant (5 % of the total volume) in the samples.

Fluorescence labelling of actin

Towards the fluorescence spectroscopic measurements the Cys^{374} residue of actin monomer was labelled with appropriate fluorophores. In the temperature dependent fluorescence resonance energy transfer (FRET) measurement the IAEDANS – IAF dyes were used as a donor – acceptor pair. To investigate the polymerization process of actin pyrene - iodoacetamide was used.

Spectrophotometry

The concentration of the examined proteins and the fluorescence labels were determined on the basis of absorption of the samples. The absorption was measured with a Shimadzu UV-2100 spectrophotometer. The concentrations of mDia fragments were determined by measuring the absorption at 280 nm in 6 M GuHCl (18), the extinction coefficients were estimated with ProtParam from the sequences (http://us.expasy.org/tools/).

The amount and the dissociation rate of the inorganic phosphate from actin filaments were measured based on the method originally described by Webb (19). The time kinetics of the absorbance of the samples at 360 nm was monitored.

Steady – state fluorescence spectroscopy

Fluorescence was measured with a Perkin Elmer LS50B spectrofluorometer equipped with a thermostated sample holder. The appropriate values of the excitation and emission wavelengths were set with monochromators. Both the excitation and emission slits were set to 5 nm.

The parameters describing the polymerization of actin (e.g.: the rate of elongation and depolymerisation) were determined from the measured pyrene transients. The critical concentration of actin was calculated from the emission spectra of pyrene labelled actin samples at different concentrations. The temperature dependent FRET measurements were carried out between 6 and 30^{o}C. The transfer efficiency (E) was obtained from the emission of donor in the absence (F_D) and in the presence of acceptor (F_{DA}) (20):

$$E = 1 - \frac{F_{DA}}{F_D} \qquad (2)$$

The intensities were corrected for the inner filter effect. To obtain more information regarding the dynamic and conformational properties of actin filaments a special FRET parameter, the *f '* parameter was calculated as follows (21):

$$f' = \frac{E}{F_{DA}} \qquad (3)$$

It was shown that the temperature dependence of the *f '* parameter is informative regarding the flexibility of the protein matrix between the donor and the acceptor molecules (21,22). In order to compare the results from different measurements we gave the relative *f '* parameter defined as the value of the *f '* parameter at a given temperature divided by the value obtained at the lowest temperature (6^{o}C).

Co-sedimentation assay

To characterise the binding of mDia fragments to actin filaments co-sedimentation assays, based on ultracentrifugation were carried out. The samples were centrifuged at 400,000 g for 30 min at 20^{o}C with a Beckman Optima MAX benchtop ultracentrifuge and a TLA-100 rotor. The protein content of the pellets and supernatants were analyzed by SDS polyacrylamide gel electrophoresis (12.5 %) and the gels were stained with coomassie blue.

The intensities of the bands corresponding to the proteins were determined with a Syngene Bio-Imaging System. The affinities of formins to the side of the actin filaments (K_d) were estimated using the following equation (23,24):

$$[A]_0 S^2 - ([A]_0 + [mDia]_0 + K_d)S + [mDia]_0 = 0 \quad (4)$$

where $[A]_0$ and $[mDia]_0$ the total actin and formin concentration, respectively. S is the fraction of bound formin calculated as the ratio of the corrected intensity of formin bands to that of the actin bands found in the pellets.

When the effect of formins on the interaction of actin filaments with cofilin was tested the actin concentrations in the supernatants (c_{actin}^{SN}) were determined from the corrected intensities of actin bands in the supernatants (B^{SN}) and in the pellets (B^{P}) with knowledge of the total actin concentration in the sample (c_{actin}) as follows:

$$c_{actin}^{SN} = \frac{B^{SN}}{B^{SN} + B^{P}} \cdot c_{actin} \quad (5)$$

Differential scanning calorimetry

The thermal properties of actin filaments were investigated using differential scanning calorimetry (DSC). The thermal denaturation of actin filaments was monitored between 0 and 100°C with a SETARAM Micro DSC II calorimeter. The calorimetric enthalpy change (ΔH) of the endothermic transition was calculated from the area under the heat denaturation curve. The enthalpy change (ΔS) was determined for the peak transition temperature (T_m):

$$\Delta S = \frac{\Delta H}{T_m} \quad (6)$$

The Gibbs free enthalpy change was calculated for $T = 22^{o}\mathrm{C}$ according to the following equation:

$$\Delta G = \Delta H - T\Delta S \qquad (7)$$

Results and discussion

The effect of mDia fragments on the polymerization kinetics of actin

The sensitivity of pyrene to the polymerization of actin was used to investigate the effect of mDia formins on the polymerization kinetics of actin (28). We found that in the presence of the core FH2 domain of mDia formins both the rate of elongation and depolymerisation decreased and the critical concentration of actin assembly increased compared to the values of these parameters characteristic for the spontaneous actin assembly. The mutations of mDia1 inhibited the effect of the core FH2 fragment on the actin polymerisation. On the basis of our results all the three monomeric core FH2 fragments due to their interaction with the barbed end of actin filaments inhibit the spontaneous polymerization of actin through a similar mechanism observed for capping proteins. The loss of function of the mutations indicates that both ends of the core FH2 domain are required for the activity. The linker region containing fragments from mDia1 and mDia3 have dramatically different effect: they accelerate actin polymerisation through their effect on the nucleation phase. The different structural features of the fragments result in different effects on actin. The flexibility of the linker region between the FH1 and FH2 domains is essential for the dimerisation of the fragments and dimerisation is essential for the nucleation of actin filaments.

The results from co-sedimentation experiments proved that all mDia fragments can bind to the side of actin filaments.

The effect of mDia fragments on the conformation of actin filaments

Temperature dependent fluorescence resonance energy transfer measurements were carried out to describe the effect of the dimeric mDia1-FH2 fragments on the protomer - protomer interactions in the actin filaments. In order to clarify whether the FRET method can be applicable to investigate the formin - actin interaction we made control experiments. The results showed that neither the temperature nor the labelling affected the activity of mDia fragments. We also found that the applied experimental circumstances did not change the critical concentration of actin assembly. These results indicate that FRET can be applied for examining the effects of mDia1 on actin filaments.

The results from FRET measurements revealed that the binding of the dimeric mDia1-FH2 to actin filaments induces conformational changes in the filaments making their structure more flexible. The effect strongly depends on the formin : actin protomer concentration ratio. The effect of mDia1-FH2 dimer increased with increasing formin concentration up to 1 : 10 = formin : actin ratio. Above this the effect of formin became smaller with increasing formin concentration. This suggests that more than one mechanism is responsible for the effect of mDia1-FH2 dimer. One can interpret the results considering the barbed-end binding (with stronger affinity) and filament side binding (with weaker affinity) mechanisms of formins (13,25), *the present work*) as follows. A formin 'cap' at the barbed end increases the flexibility of the filament through long range allosteric interactions, while formins bound to the side of the actin filaments as formin 'cramps' linking actin protomers along the sides of filaments can stabilise the molecular interactions between neighbouring protomers and make the filaments stiffer (*Figure 1.*). The superposition of these two effects was observed in the measurements.

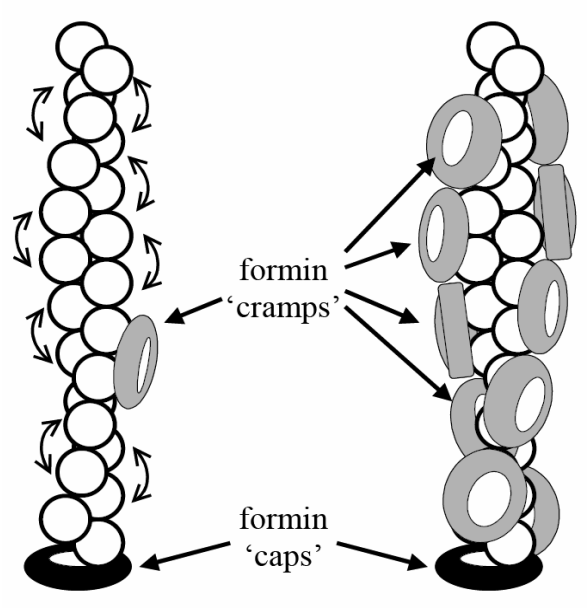

Figure 1.: The schematic representation of the proposed model for the formin - actin interaction. Actin protomers in the filaments are indicated with open circles. The formin fragments bound to the barbed end (formin caps) and to the side of the actin filaments (formin cramps) are represented by black and grey ellipses, respectively. The left side of the figure shows the situation at low formin : actin stochiometry while the right side of the figure corresponds to high formin concentration.

We found that the monomeric mDia1 fragment induced increase in the flexibility of actin filaments but the magnitude of the loosening was smaller than in the presence of the dimeric fragment and did not show such concentration dependence. These observations support our model describing the effect of mDia1-FH2 dimer.

The nature of the formin actin interaction

We showed that the effects of formins on the polymerisation process and the conformation properties of actin filaments depended on the ionic strength: the effect was smaller applying higher ion concentration than smaller ionic strength. Similar ionic strength dependence of the affinity of the side binding of formins could be observed. We assume that higher ionic strength results in the loosening of the binding of formins to actin filaments. This observation can imply that the electrostatic interactions play important role in the interactions of the two proteins. The weakening of the electrostatic interactions between protein groups resulted from the effect of solvent causes the weaker binding of formin to actin.

The effect of mDia fragments on the functional properties of actin filaments and their interaction with cofilin

In order to obtain more detailed information regarding the mechanism of formins we investigated whether the functional properties of actin filaments were modified in the presence of formins. The results showed that actin filaments were thermodynamically less stable and the dissociation rate of the inorganic phosphate from filaments increased in the presence of formins. These observations suggest that there is direct correlation between the flexibility and the functional properties of actin filaments.

The formin induced change in the conformation of actin filaments was accompanied by modification in their interaction with cofilin. We found that cofilin depolymerises more effectively the formin nucleated actin filaments, which could be due to the destabilisation effect of formins on the actin filament structure.

The biological relevance of our research: a possible new mechanism in the regulation of actin cytoskeleton

In living cells the actin structures can be classified on the basis of their morphology and intracellular localization. The different actin networks play role in different functions of cells. In many cases the proteins involved in these complexes can be attributed to specific actin nucleation factors. In cells a group of proteins localize to formin-nucleated actin structures (e.g.: tropomyosin (27)), while other proteins are typically associated with actin filaments nucleated by the Arp2/3 complex (eg: ADF/cofilins (26)). However how the formation of these protein complexes is regulated is unknown.

The present work provides evidences that formin fragments can have substantial and allosteric effects on the conformation of actin filaments by binding to the barbed end. According to our hypothesis due to the conformational changes induced by formins by their binding formins can change the affinity of actin binding proteins to actin filaments and thus determine which proteins are involved in the corresponding complexes.

Based on this observation one can propose a special mechanism of the regulation of the formation of cytoskeletal protein complexes. In consequence of their function, actin nucleation factors are the first to bind to the newly generated actin filaments. In general one can envisage that the nucleation factors can modify the conformation of actin filaments by binding to them, which play an essential role in determining the affinity of actin binding proteins to actin filaments. The nucleation factors can change the affinity of actin binding proteins to actin filaments and in this way regulate the formation of protein complexes. By reason of this hypothesis the actin filaments are not only passive cables rather serve regulatory informational channels in living cells.

Synopsis

In the present research we found, that:

- The monomeric core FH2 domains from mDia proteins by binding to the barbed end of the filaments inhibit the assembly of actin filaments.
- Both ends of the core FH2 domain are required for its activity.
- The linker region containing fragments can accelerate actin polymerisation by catalysing the nucleation phase.
- The different effects result from the structural differences: the linker region is essential for dimerisation and dimerisation is essential for nucleation.
- The dimeric mDia1-FH2 modifies the dynamic properties of actin filaments: a formin ‘cap’ bound to the barbed end increases the flexibility of actin filaments, while formin ‘cramps’ bound to the side of the filaments can stabilise their structure.
- The functional properties of flexible actin filaments nucleated by mDia1-FH2 dimer are modified:
  - the thermodynamic stability of actin filaments decreased,
  - the rate of phosphate dissociation from actin filaments increased,
  - and the interaction of actin filaments with cofilin was modified.

The publication the thesis is based on

Bugyi B, G. Papp, G. Hild, D. Lőrinczy, EM. Nevalainen, P. Lappalainen, B. Somogyi, M. Nyitrai: Formins regulate actin filament flexibility through long-range allosteric interactions. *Journal of Biological Chemistry*, **281.**, 10727-10736, 2006. IF: 6.355

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Lectures

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Bugyi B., G. Papp, Z. Ujfalusi, Sz. Barkó, M. Nyitrai and B. Somogyi. Formin caps and cramps on actin filaments: A possible mechanism to regulate the formation of cytoskeletal protein complexes. 2005. September 25-28., 8th International Symposium on Instrumental Analysis, Graz, Austria.

Posters

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Bugyi B., G. Papp, Sz. Barkó, Z. Ujfalusi, M. Nyitrai: The effect of formin homology domain 2 on the dynamic properties of actin filaments, XXII. Congress of the Hungarian Biophysical Society, 2005. June 26-29., Debrecen, Hungary.

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*: these authors contributed equally to this work

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