The role of nucleotides and actin bundling proteins in the functional conformation changes of actin

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Introduction

Actin is one of the main component in eukaryote cells which plays significant role in many cellular processes, like force-generation, maintenance of the shape of cells, celldivision cycle, and transport processes. Many structural investigations showed that the monomer actin (G-actin) consists of four subdomains, the internal structure of subdomains are stabilized by nucleotide, either ATP or ADP and a divalent cation (calcium or magnesium). The structure of subdomains is dynamic, as derived by several biophysical and structural studies; the subdomain might have different motional states depending on the bound cations and nucleotides. Site-specific cross-linking among F-actin monomers inhibits the motion and force generation in myosin. The ability of G-actin to reversible polymerize into long filaments is essential for many cellular functions.

The structure of monomer and polymerized actins, ATP and ADP-actins were mainly obtained from crystal structures of actin and derived from interactions with different proteins. The detailed investigations concluded that ATP-actin monomers and ADP-actin monomers were structurally different, and they exhibited nucleotide-dependent internal motion in polymerized form. Similarly, structural differences were observed in the nature of the tightly bound divalent cations Ca^{2+} and Mg^{2+}. The nucleotides and the cations are located in the cleft in the center of the molecule. The cleft might be in open or closed conformation, in which the two larger domains are twisted relative to each other. The opening of cleft affects the binding mode of nucleotides, and might be in relation with the nucleotide release. The exchange of the nucleotide and/or the cations affects the structure of subdomain-2 and the C terminal region of actin. The different functions of actin require special internal organization in time and space, and special interactions with other molecules at well-defined regions.

There are several actin filament bundling proteins that mediate diverse processes in cells such as the formation of cell membrane protrusions or cell adhesions and stress fiber based locomotion. Among the various cross-linkers alpha-actinin and fascin are the most abundant ones. Their interaction with actin is different and probably adapted to the related specific biological functions.

The globular fascin (55-58 kDa, $\sim$ 5 nm diameter) has an evolutionarily highly conserved amino acid sequence folded up into four beta-trefoil domains. There are three isoforms of fascin in mammals; fascin-1, 2 and 3 and they are expressed in different

tissues. While in many cases phosphorylation activates proteins and signaling pathways, fascin is one of the actin binding proteins that is negatively regulated by phosphorylation. Fascin has two major F-actin binding sites, one in the N terminal (β trefoil-1) and another in C terminal (β trefoil-3) position, and these binding sites assist the formation of stable and relatively rigid actin bundles. These actin bundles serve as highways of vesicle transport from the cell body to the constantly developing leading edge of the cell protrusion. Fascin localizes to filopodia, microspikes and actin-based protrusions underneath the plasma membrane and is involved in the assembly and maintaining of the parallel bundled F-actin structure at the filopodia tips in motile cells. Recently it has been shown that fascin can modulate the conformation and dynamic properties of actin filaments.

Alpha-actinin is an antiparallel homodimer (20-30 nm head to head length) with a single N-terminal actin-binding domain followed by four spectrin-like triple helical motifs arranged in tandem structure. The spectrin motifs are responsible for non-covalent dimer formation and the specific features of the dimerization domains defines the head-to-head length. The orientation of the actin-binding heads is important in actin binding and consequent bundling. Alpha-actinin can be found at many different sites in cells, such as Z-disks of skeletal muscle sarcomers, and in non-muscle cell adhesion plaques or at the leading edge of motile cells.

Despite of their similar (crosslinking and bundling) basic functions alpha-actinin and fascin bound actin filaments display different mechanical properties. There are differences in the dependence of elasticity and rate of bundle assembly on linker protein concentration. The most evident difference is that according to the size of these molecules and the distance between two actin binding domains the formed actin network is different. Fascin forms tight bundles in contrast with alpha-actinin. However, the correlation between the characteristic geometrical properties and their functional behavior is not necessarily simple. Both fascin associated tight actin bundles in microspikes and stretch fibers formed by alpha-actinin bundling are permissive for motility of myosin motors. In fascin mediated bundles myosin heads can bind to the bundles the same way as to individual actin filaments and the in vitro motility assays have shown no difference in actin filament and bundle sliding speed on myosin covered plates at relatively high, 1 : 2 fascin : actin concentration ratios.

Aims

In the first part of my work the ATP nucleotide in monomer actin was exchanged by its non-hydrolyzable analogue adenylyl-imidodiphosphate (AMP.PNP), and using two biophysical methods, electron paramagnetic resonance spectroscopy (EPR) and differential scanning calorimetry (DSC), we studied the local and global changes in globular and fibrous actin following the nucleotide exchange.

Our questions were:

(1) How changes the actin dynamics in case of other nucleotides?

(2) How changes the rotational dynamics of F-actin, if the monomers binds ATP and not ADP?

In the second part of my work reported here provided information regarding the fascin and alpha-actinin induced molecular changes in actin filaments on two distinctly different time scales, on the nanosecond and on the microsecond time-scale.

Our questions with these experiments were:

(3) What are differences between the rotational dynamics of the fascin and the alpha-actinin made actin bundles?

(4) What are the effects of the bundling proteins to the structure of the actin protomers?

(5) What are the effects of the connections between the filaments to the dynamics of the actin filaments?

Materials and Methods

Protein purification and modification.

Actin was purified from the domestic white rabbit skeletal back and leg muscles and was stored in G-actin buffer (0.2 mM ATP, 0.1 mM CaCl2, 4 mM TRIS or MOPS, pH 8.0, 0.1 mM DTT and 0.005% NaN3) at 4 °C. The actin monomer (G-actin) concentration was determined from absorbance measured at 290 nm using a molar extinction coefficient of 0.63 mg-1 ml cm-1 using a Shimadzu UV2100 spectrophotometer. Actin filaments (F-actin) were polymerized by the addition of 2 mM MgCl2 and 100 mM KCl (final concentrations) to G-actin buffer for 2 hours at room temperature and then kept on ice until use.

Fascin and *alpha*-actinin were extracted as recombinant proteins from cells of E. coli expression system. Cells were thawed at 4 °C in PBS (proportional to the amount of wet cell mass), protease inhibitor mix, PMSF, and lysozyme was added to the cells. Then the cells were disrupted by handheld homogenizer and sonicator (5 x 1 minute pulses of 80 % amplitude). To the cell suspension DNase I enzyme was added and then stirred for 1 hour at 0 °C. After the incubation the excess cell debris were separated from the sample by ultracentrifugation (30,000 rpm, 4 °C, 30 min).

The proteins (GST-fusion tagged recombinant alpha-actinin and fascin) were purified with affinity chromatography (Pharmacia FPLC), in which the GST-protein was bound to the beads of the GSH (glutathione - sepharose 4B) column. To remove nonspecifically bound proteins we used 20x column volume of washing buffer (50 mM Tris / HCl (pH 7,5), 300 mM NaCl, 3 mM DTT, 1 mM EDTA). After cleavage with Factor Xa (0 ° C, 16 h) the protein was eluted in buffer (10 mM Tris / HCl (pH 7,5), 150 mM NaCl, 3 mM DTT, 1 mM EDTA). The sample was concentrated in Amicon Ultra 10 MWCO centrifuge tubes using Janetzki K26 centrifuge (4000 rpm, 4 °C). The remaining contaminating proteins were removed by gel filtration (Superdex G-75 column). Samples were taken from the fractions corresponding to each absorbance peak and then purity was checked on SDS-acrylamide gel. The fractions containing the protein of our interest were collected and concentrated as described earlier. For storage our protein were dialyzed against F-actin buffer, and frozen in liquid nitrogen then stored at -80 °C.

The bundling activity of the proteins was tested with fluorescent labeled (5-10%)

actin using microscopy. The previously polymerized actin was mixed with the bundling protein at around 100:1 actin : bundling protein concentration ratio then the appearance of bundled filaments were verified.

Spin labelling of actin.

Actin was spin-labelled with N-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl)-maleimide spin label (MSL) in filamentous form in excess of MSL (a molar ratio of 1:1.2) for 12 hours at 4 °C. Unreacted spin labels were removed by pelleting the actin by ultracentrifugation, and then pellet was resuspended, homogenized and dialyzed in G-actin buffer. Labelling ratio has been determined by comparing the double integral of the EPR spectra measured at given actin concentrations and a known concentration of MSL prepared in G-actin buffer. EPR spectra for both cases were measured under identic spectrometer settings, unless receiver gains were taken into account during the calculations. The range of the labeling ratio (labelled protein to whole protein concentration), thus determined for different samples has been found to be between 0.8 - 0.9.

EPR spectroscopy.

Conventional EPR spectra of actin were recorded with an ESP 300E X-band spectrometer (Bruker Biospin, Rheinstetten, Germany). First harmonic in-phase, absorption spectra were obtained using 20 mW microwave power and 100 kHz magnetic field modulation with amplitude of 0.20 mT; receiver gain, conversion time, time constant and scan numbers of 8.93•104, 81.9 ms, 10.24 ms and 25 scans were used, respectively. Actin concentration varied between 80-120 μM. In saturation transfer (ST) EPR measurements the second harmonic out-of-phase spectra were recorded (50 kHz magnetic field modulation, 0.5 mT modulation amplitude and 63 mW microwave power, 32 or 64 scans with receiver gain, conversion time, time constant and scan numbers of 8.93·104, 81.9 ms, 10.24 ms). For ST-EPR measurements only samples with the highest molar ratios of bundling proteins to actin (1 : 20) were used. The temperature was set to 20 °C with a temperature controller. The protein samples were placed in two capillary tubes (Mettler ME-18552 melting point tubes), each of them contained 10 μl solution. The

sample tubes were positioned parallel in the center region of the TM 110 cylindrical cavity. A small thermocouple was inserted in one of the capillary tubes, and the temperature was regulated with a diTC2007 type temperature controller. The EPR spectra was evaluated with the WINEPR program from Bruker and with a computer program developed in our laboratory.

Evaluation of the EPR spectra.

In order to characterize the changes in conventional EPR spectra we determined the distances between the two outer extremes that is characteristic of the rotational motion of the spin labelled molecules. In case of molecules which are nearly spherical ones, one can also use the formula introduced by the Freed's group for spherical tops, if the rotational correlation times are in the range not longer than about 10-7 s. Although this condition is fulfilled by the G-actin, it is not correct for F-actin molecules, especially if they are bundled by the fascin or alpha-actinin. (We have tried to make EPR spectral simulations too, however the results pointed out that neither this spectral simulations allows a correct determination of the rotational correlation time. The rotational motion of the bundled F-actin molecules are beyond the time range where the slow motional simulation program, used earlier also by us for other systems would allow a correct determination of the rotational correlation times. Evaluating the ST-EPR spectra the usual L''/L and the C''/C parameter were determined from the spectra. Correlation times in the ST-EPR time domain has been calculated based on the digitized values from the tables given for MSL-labeled hemoglobin according to Hyde and Dalton.

DSC measurements

Thermal unfolding of actin was monitored by SETARAM Micro DSC III calorimeter. All experiments were performed between 20 and 100 °C, the heating rate was 0.3_K/min in all cases. We used conventional Hastelloy batch vessels for the experiments with 800 µl sample volume. The sample and reference vessels were equilibrated with aprecision of 0.1 mg. Buffer A solutions (0.2 mM ATP,0.1 mM CaCl2, 4 mM MOPS, pH 8 or the same buffer A without ATP) were used as a reference sample. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve.

Simple mathematical calculations were used to obtain the thermodynamic data of samples (excess heat capacity Cp,ex,transition temperature Tm and calorimetric enthalpy change DH).

Results

In the first part of my work the ATP nucleotide in monomer actin was exchanged by its non-hydrolyzable analogue AMP.PNP, and using two biophysical methods, EPR spectroscopy and DSC, we studied the effects of the changes to the flexibility of the filament.

Our observations were:

(1) The AMP.PNP bound globular and filamentous actins are thermodinamically more stabil compared to the ATP-and ADP-actins.The nucleotide exchange leads to the decreased internal flexibility of the filament. The ATP-hydrolisis may change the conformation of the actin monomers during filament formation which might contribute to the change of flexibility of the actin filaments.

(2) In F-forms of actins only a small changes was detected on AMP.PNP-F-actin in comparison of ADP-F-actin. The flexibility of filaments seems to be essential for the proper function of F-actin in biological systems.

In the second part of my work we observed the conformational and dynamical changes caused by the alpha-actinin and fascin in the structure and flexibility of the actin filament. Our experiments reported here provided information regarding the fascin and alpha-actinin induced molecular changes in actin filaments on two distinctly different time scales, on the nanosecond and on the microsecond time-scale. The faster motions are attributed in actin to the motions of actin subdomains, protomers or short segments of actin filaments. The longer time-scale motions are more characteristic for the correlated motion of the entire actin filaments, including bending and torsional modes.

(3) We found that slower bending and torsional motions were restricted after the binding of these bundling proteins and the core structural elements of the actin filaments became more rigid. On a faster, nanosecond time scale molecular motions the two proteins were found to induce opposite changes in the actin filaments.

(4) The core structure of the actin filaments was stabilized providing a more rigid structural framework for biological functions. Considering that in this respect similar tendency was observed with the two cross-linking proteins the stabilization may simply be the effect of the formation of bundles, in which the motion of the actin filaments is restricted by their actin filament neighbors.

(5) Saturation transfer EPR experiments showed that the larger scale bending and torsional flexibilities along the actin filaments followed decreasing tendency after the binding of either fascin or alpha-actinin and we conclude that the filaments became more rigid in these complexes. The core structure of the actin filaments was stabilized providing a more rigid structural framework for biological functions.

Publications

Publications related to the thesis:

Katalin Türmer, Franciska Könczöl, Denes Lőrinczy and Jozsef Belagyi: AMP.PNP affects the dynamical properties of monomerand polymerized actin: A DSC and an EPR study 2011; Journal of Thermal Analysis and Calorimetry Vol. 108 95-100.

IF: 2,09

Katalin Türmer, József Orbán, Pál Gróf, Miklós Nyitrai: Fascin and alfa-actinin can regulate the conformation of actin filaments. 2015 Biochimica et Biophysica Acta (BBA) - General Subjects, 1850, (9), 1855-1861.

IF: 3,66

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IF: 4,702

Total IF: 12,622