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Molecular analysis of microorganisms' life processes

The effect of formins on conformational and dynamic properties of actin filaments

Ph.D. thesis

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Introduction

The cytoskeleton is a network of the cytoplasm of eucaryotic cells constituted by filamentous and tubular structures. As the cytoskeleton is contacted to cell membrane and to other cell components, it gives a spacial frame to the molecular events of physiological processes. Fundamentally the cytoskeletal network of the eucariotic cells is composed by three filamentous structures, these are the systems of microtubules, microfilaments, and the intermediate filaments.

The dynamic reorganization of microfilaments playes important role in such critical processes in the cell as the endocytosis, cell division, cell morphogenesis, or the determination of cell polarization. The coordination of these intracellular processes are due to the work of 20-30 highly conserved actin binding proteins and many signaling upstream molecules. The combined activity of these factors perfectly regulate the spatial and temporal organization of actin structures, and ensure the reorganization of cytoskeleton according to cell affecting extra- and intracellular stimuli.

Actin is the main component of the microfilamental network. It occurs in two forms both *in vivo* and *in vitro.* G-actin is the globular form, which actually is the actin monomer, the other is derived from the bound actin monomers and constitutes a polimerized filamental form. The monomer contains two domains, in between of the domains in the cleft cation-binding (Mg^{2+}, Ca^{2+}) and nucleotide binding sites (ATP, ADP, ADP+ P_i) are present. The actin filament formation can be devided to three major phases. Starting with the so-called nucleation when some (two or three) actin monomers bind to each other. The evolved nucleus starts the second phase, which is the diffusion-controlled elongation, where other monomers join to the filament. The third phase can be characterized with a dinamic equilibrium, where the association and dissociation occure at both ends of the filament, however the incorporation of monomers is more typical at the barbed end, whilst the dissociation of monomers is more frequent at the pointed end. Forasmuch these processes happen with different kinetics at the two ends of the filament, therefore the length of the filament does not change, but inside the filament a continous internal movement occurs (treadmilling).

The formation of the actin filaments, and the spatial position correlated to each other is determined by numerous actin binding proteins. Due to the interactions with other proteins, the conformational state of the filament changes and the caused effect modifies the mechanical properties of the filament.

The functional attributes of actin monomer and polimer is affected by numerous agents, such as different metal- and other ions, the hydrolitic state of the bound nucleotide, different drogs and actin binding proteins. The regulated and rapid actin nucleation depends on actin nucleating factors, which form three groups. The representative of the first group is the Arp2/3 complex, which binds to the side of the filament, and mymicking the plus end of actin filament could form a branching

filament. The branched filaments then result the formation of the actin network. The second group composed by the Spire proteins, which could form a prenucleation complex derived from four actin monomers. This complex plays as the origin of the filament elongation. The third group is composed by formins, that form unbranched filaments.

Formins have important role in the reorganization of actin structure of the cytoskeleton. Formins can be found in numerous eukaryotic organism, and three highly conserved regions has been determined: the „formin homology" FH1, FH2 and FH3 domains. Among the several formin families mostly the best characterized are the „Diaphanous-related" formins, these formins – apart of the above nominated conserved domains – also have different regulatory regions. The funcional form of formins is the antiparalelly bound FH2 dimer, which possesses actin nucleating ability. The formation of FH2 dimer requires a linker region at the N-terminal of the FH2 domain. This linker links the FH1 domain to FH2 domain, and performs the flexibility of the dimer.

Aims

In the first part of our work we planned the preparation/purification of the mDia1-FH2 fragment, therefore we optimized the expression system according to the capacity of the laboratory facility.

In the further part of our work our aim was to investigate formin induced dynamic changes of actin filaments by fluorescence spectroscopy methodologies. In our focus was the examination of different conditions that affect the conformational changes and flexibility of the actin filaments after binding of formin.

- We planned to get the answer, how affects the mDia1-FH2 the fluorescence lifetime of IAEDANS fluorophore-labeled actin filaments.
- Our aim was to study the formin concentration dependence of actin filament flexibility. For the detection of changement of the flexibility we would like to demonstrate by changes of lifetime of fluorescence decay of IAEDANS F-actin.
- Meanwhile our investigations a new question occured, whether the total formin concentration or the formin:actin concentration ratio determines the determined formin-effect.
- Our previous experiments demonstrated that the ionic strength of the environment of actin filaments determines the conformation of the filament. We would find the answer whether the alteration of the ionic strength affects the formin induced changes in the flexibility of the filaments.

Methods

The preparation of proteins

The first step of actin preparation was the actin-aceton powder preparation from rabbit (*Oryctolagus cuniculus*) skeletal muscle. The isolation of actin from it was performed according to Spudich and Watt, and stored and diluted in buffer A (4 mM TRIS-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT) to achieve the necessary concentration.

The mammalian (*Mus musculus*) mDia1-FH2 proteins were expressed as GST-fusion proteins in prokaryotic cells. The DNS fragment containing pGEX-4-T3 plasmid was transformed into *E. coli* BL21 (DE3)pLysS competent cells. After the expression, the GST-mDia1-FH2 was separated by affinity chromatography, and purified by size exclusion chromatography, then the protein was concentrated. The concentration of the fragment was determined photometrically.

The G-actin was polimerized with 10 mM KCl, and 0.5 mM $MgCl_2$ (noted if otherwise).

The sample contained formin fragments that were added to the solution prior to the start of polimerization. To exclude the effect of the storing buffer of formin, its volume regardless of whether it was added or not, but with or without formins was kept constant (5% of total volume) in the samples.

Fluorescence labeling of actin

To perform actin polimerization assays actin was labelled by pyrene-iodacetamide. For the fluorescence lifetime and anisotropy measurements the Cys^{374} residue of actin monomer was labeled with IAEDANS dye. The ratio of labeling was determined photometrically.

Fluorescence lifetime and anizotropy measurements

Fluorescence lifetime and anizotropy measurements were carried out with ISS K2 multifrequency phase fluorimeter. The evaluation of fluorescence lifetime measurements was analyzed using both two-exponential decay model and Gaussian distribution, which were selected according to χ^2-probe analysis.

To define the fluorescence lifetimes a double exponential lifetime fit was applied, where the two fluorescence lifetimes were determined according to the following equation:

$$\tau_{aver} = \frac{\alpha_1 \tau_1^{\,2} + \alpha_2 \tau_2^{\,2}}{\alpha_1 \tau_1 + \alpha_2 \tau_2} \qquad (1)$$

where the τ_{aver} is the average fluorescent lifetime, $\alpha_{1,2}$ and $\tau_{1,2}$ are the amplitudes and lifetimes.

The anisotropy decay measurements

The anisotropy is expected to decay as a sum of exponentials. The raw data were fitted with the following double exponential function:

$$r(t) = r_1 e^{-t/\phi_1} + r_2 e^{-t/\phi_2} \qquad (2)$$

where ϕ_1 and ϕ_2 are the two rotational correlation times with amplitudes r_1 and r_2, respectively.

The limiting anisotropy recovered at zero time is given by

$$R_0 = r_1 + r_2 \qquad (3)$$

Assuming that the two-dimensional angular range, Θ, within which the fluorophore performs wobbling motion could be related to the amplitudes of the two rotational modes according to the following equation:

$$\frac{r_1}{r_1 + r_2} = \frac{\cos^2 \Theta (1 + \cos \Theta)^2}{4} \qquad (4)$$

Here, r_1 is the amplitude of the slower, r_2 is the amplitude of the faster of the two exponential decay components. The determined half angle is correlated to the flexibility of the protein matrix in the environment of the fluorophore, consequently it is an appropriate parameter to describe the dynamic properties of the microenvironment of the fluorophore.

Results and discussion

The effect of mDia1-FH2 on the fluorescence lifetime of IAEDANS-actin

Fluorescence probes attached to proteins can reflect the conformational changes in their microenvironment. In our experiments, the fluorescence lifetime of IAEDANS attached to Cys^{374} was investigated to characterize the effect of mDia1-FH2 on the structure of actin filaments. In these measurements, 20 μM actin was polymerized in the presence of various concentrations of mDia1-FH2 (0–5 μM).

The average lifetimes from double-exponential analyses (τ_{aver}) (Eq. 1) and the mean fluorescence lifetimes from Gaussian analyses were used to interpret of the fluorescence lifetime results. Both types of analyses showed that the fluorescence lifetime of IAEDANS decreased from 20 ns in the absence of formins to 18.5 ns in the presence of mDia1-FH2. The decrease of the fluorescence lifetime was formin concentration-dependent below 500 nM mDia1-FH2 and became formin concentration-independent above this concentration. The effect of formin on the fluorescence lifetimes indicated that the binding of mDia1-FH2 modified the microenvironment of the Cys^{374} in actin filaments.

The effect of mDia1-FH2 on the dynamic properties of actin filaments

To further characterize the formin-induced conformational changes in actin filaments the anisotropy decay of the IAEDANS-actin filaments was measured between 2 MHz and 100 MHz. The obtained frequency-dependent phase and modulation data were analyzed with double-exponential fits (Eq. 2). In these analyses, two rotational correlation times were determined. We attributed the shorter rotational correlation time (ϕ_2) to the motion of the probe relative to the protein, but the longer one (ϕ_1) to the motion of the protein matrix. The former one ranged between 2 ns and 4 ns in our experiments and showed little formin-concentration dependence. The longer rotational correlation time was 700 ns in the absence of formin, similar to that observed in previous works. The value of this parameter decreased to 100 ns in the presence of 500 nM mDia1-FH2 and remained formin-concentration-independent above this concentration. Previously it was established that the value of the longer correlation time is inversely proportional to the flexibility of the actin filaments. Therefore, the formin-induced decrease of the rotational correlation time suggested that the interactions between neighboring protomers became weaker, and the actin filaments became more flexible, due to the binding of the mDia1-FH2.

The amplitudes of the rotational correlation times (r_1 and r_2) were also found to be formin-concentration-dependent, indicating that the relative contribution of the two rotational modes to the anisotropy decay was modified by the binding of formin. The value of R_0 (Eq. 3) was smaller in the presence of mDia1-FH2 than in the absence of it. The formin dependence of this parameter was well pronounced below 500 nM mDia1-FH2, where R_0 decreased to 0.265, whereas its value did not change above this concentration. The decrease of the R_0 was attributed to the less rigid protein structure around the bound fluorophore and reflected the loosening effect of mDia1-FH2 on the microenvironment of the Cys^{374}.

The semi-angle (Θ) of the cone within which the IAEDANS at Cys^{374} performs wobbling motion was calculated using Eq. 4. The value of the semi-angle was larger in the presence of mDia1-FH2 than in the absence of it and followed formin-concentration dependence similar to that of the longer rotational correlation times and that of the R_0.

The greater semi-angles observed in the presence of formin corroborated our conclusions from the formin dependence of the fluorescence lifetimes and R_0 that the microenvironment of the fluorescent probe was more flexible when the filaments were polymerized with mDia1-FH2.

The actin-concentration independence of the formin effect

The fluorescence parameters obtained at 20 mM actin were formin-dependent below 500 nM mDia1-FH2 and became formin-independent above this concentration. In the next set of experiments, we investigated whether it was the formin:actin concentration ratio (1:40), or the total formin concentration (500 nM) that was responsible for this observation. Above 500 nM the formin-concentration dependence of the fluorescence parameters became negligible. The actin concentration independence of the smallest formin concentration at which the largest formin effect was observed suggested that the total formin concentration determined the appearance of the maximum formin effect, not the formin:actin concentration ratio.

The ionic-strength dependence of the effects

In the absence of formins the value of the longer rotational correlation time was independent of ionic strength. The binding of mDia1-FH2 to actin filaments resulted in the decrease of the longer rotational correlation time. At this higher ionic strength, the formin induced change of this parameter was only half of that observed at lower salt (0.5 mM $MgCl_2$ and 10 mM KCl). We assume that in the formation of actin formin interactions have some important roles of electrostatic

interactions between the molecules. At higher ionic strength presumably evolves weaker binding, because the effect of vehicle shields the electrostatic interactions between different proteins.

The relevance of our results in the regulation of actin cytoskeleton

Formins, as actin nucleating factors primarily get in contact to the newly formed actin filament. It possibly may be determinant the altering effect of binding of nucleation factorts in the regulation of temporal and spatial formation of certain protein structures. The allosteric interactions might cause modification in the structure as a consquence of binding. Nevertheless the nucleation factors bind to certain point of the filament, it changes the conformation of the whole filament. From that establishment, moreover then actin filaments are structural elements of the cell, can serve as informational channels in the regulation of the actin cytoskeleton.

Summary

Our aim was the detailed examination of mDia1-FH2 induced effect to the flexibility of actin filament. At first step the optimization of the protein expression system to the local circumstances has occured. Beside the fitted preparation steps to the available tools and facilities it resulted in a more efficient protein expression.

- The results exhibited, that the lifetime of the fluorescent dye bound to the filament decreased in the presence of mDia1-FH2, indicating an alteration in the microenvironment of the fluorescent dye.
- The anisotropy decay measurement supported, that formin influences the segmental motion of actin filaments. Due to the weakening of the physico-chemical interactions of the neighboring protomers, the flexibility of the filament increased. This change proved to be formin dependent until 500 nM mDia1-FH2 concentration, but above this concentration it was shown to be independent from it. All these facts suggests that the binding of formin to the barbed end of the filament caused conformational changes in the whole filament, or in a segment of the filament.
- We proved that the flexibilizing effect of formin is independent from the concentration of actin. We could determine the minimal range of one formin molecule's effect based on the applied actin : formin ratios. According to these results one formin molecule bound to the barbed end of the filament is able to affect the flexibility in the range of – at least – 160 protomers. This change manifested in the increment of the flexibility of the whole filament, and were caused by long-range allosteric interactions.
- We/It has been demonstrated also the ionic strength dependence of formin effect. Based on our investigations, the effect of formin proved to be smaller at high ionic strength.

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