The effect of tropomyosin and heavy meromyosin on the flexibility of formin-nucleated actin filaments

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1 General introduction

1.1 Actin

Actin plays indispensable roles in several processes in eukaryotic cells. The dynamic actin network is essential for the determination and regulation of cell shape, polarity, cell division and the transport processes within eukaryotic cells. Actin was discovered by a then young biochemist, Brunó Straub, in the laboratory of Albert Szent-Györgyi in 1942. (Further information: http://actin.aok.pte.hu/archives/)

Actin is the main component of the microfilament system, and it contributes 15 % to the total protein content of skeletal muscle. Its molecular weight is 42.3 kDa and it is composed of 375 amino acids. In cells there are two forms of actin, the monomer or globular (G-actin) and the polymer or filament (F-actin) forms. The filaments build up from monomers linked by non-covalent bonds. The polymerization of the actin has three main phases. The first phase starts with the activation of monomers followed by the nucleation where two or three monomers bind to form a nucleus. This nucleation step is relatively slow because this process is thermodynamically unfavourable which also explains the instability of the dimers and trimmers. The next phase of the polymerization is the diffusion-controlled elongation where monomers incorporate into the forming filament. In the last stage a dynamic equilibrium, the so-called 'treadmilling' is dominant. In this phase there is association and dissociation on both ends of a filament but the kinetics at each end are different. On one end of the filament monomers mainly incorporate to the filament (association) while on the other end they rather detach from the filament (dissociation), resulting in a filament length that does not change. One can say that actin filaments are polarized; they have a dynamically growing positive end and a negative end where the dissociation of the protomers is the main process. The protomers at the growing end bind ATP, while the protomers in the shortening end bind ADP because of the hydrolysis of ATP.

1.2 Formins as actin assembly factors

Formins are conserved proteins in eukaryotes during the evolution. They can be identified by the presence of a highly conserved region, the so-called formin homology domain 2 (FH2). Varieties of formins act through the actin and microtubule systems and take part in meiosis, mitosis, cell movements, cell polarity, filopodia formation, embryonic development, sperm acrosome formation, endocytosis, etc.. The biochemical effect of formins on actin is strong, typically requiring only 5-200 nM formin for robust nucleation and/or elongation activity *in vitro*. These multi-domain proteins are present in many animal and plant species. Numerous formin members are organized into families. Proteins of formin families play a very important role in the organization and rearrangement of the cytoskeleton.

According to the model of Zigmond, the FH2 domain is necessary and sufficient for the nucleation of actin and for the stabilization of actin dimers. Once a filament is nucleated, the dimeric FH2 domain moves processively with the growing positive end, protecting it from capping proteins, while allowing the rapid addition of new subunits.

Formins exert their greatest effect mostly on the actin cytoskeleton, which facilitating the formation of long unbranched filaments.

1.3 Tropomyosin

Tropomyosins (Tm) form a large family of protein isoforms, expressed from multiple genes. They are components of the thin filaments, bind to the alpha-helical groove of the actin filament and play a very important role in the regulation of muscle contraction. In non-muscle cells Tm isoforms have been implicated in several processes including cytokinesis, vesicle transport, motility, morphogenesis and cell transformation. Since it was discovered in 1948 its role in the muscle contraction has been published many times, though the exact functions of tropomyosins in non-muscle cells have not exactly been determined yet.

1.4 Myosin and heavy meromyosin

The term myosin is used to refer to a diverse superfamily of molecular motors capable of translocating either actin filaments or other cargo on fixed actin filaments. The myosin superfamily consists of at least 35 different classes. Although their contractile activity is most evident in differentiated muscle tissues, it is also observed in nonmuscle cells in diverse

cellular processes such as cell division, cell migration, and cell-cell or cell-matrix adhesion.

The proteolytic digestion of myosin II by α-chymotrypsin results in the so-called heavy meromyosin (HMM) that is commonly used in experimental work because of its solubility. It consists of two motor domains which dimerise through an α-helical coiled coil.

2 Main objectives

It was shown by fluorescence spectroscopy that formin fragments have the ability to increase the flexibility of actin filaments through long-range allosteric interactions after binding to the positive end of the filaments. These studies underlined the importance of the intramolecular conformational changes induced by formins in the structure of actin filaments. The generation of flexible actin filaments by formin binding can have a well-defined biological role, which is manifested under special intracellular conditions for particular functions. My aim was to find actin-binding proteins, which may have the ability to affect the formin-induced flexibility of actin filaments.

Formin-generated actin structures interact with many actin-binding proteins that can influence the formin-induced conformational transitions. One of these interacting proteins, tropomyosin, was shown to reverse the formin-induced conformational changes and stabilize the structure of the filaments. Myosin is one of the most abundant actin-binding proteins that also localizes to formin-nucleated actin structures in cells. Myosin binding to actin filaments induces long-range allosteric and cooperative effects in the conformation of the filaments, which was shown to be dependent on the myosin isoform. Thus, myosin can be another candidate for the regulation of the conformational dynamics of formin-nucleated actin structures. For these purposes fluorescence spectroscopy methods were applied. Our aim was to answer the following questions:

- How do the formin fragments influence the structure of actin filaments?
- What is the effect of tropomyosin on the flexibility of mDia1FH2-induced actin filaments?
- Is the effect of tropomyosin ionic strength dependent?
- Does heavy meromyosin affect the flexibility of formin-nucleated actin filaments?

3 Experimental methods

3.1 Protein preparation and purification

Actin, mDia1-FH2 formin fragment, tropomyosin and heavy meromyosin were prepared based on previously described methods.

3.2 Fluorescent labelling of actin

Actin was labelled with IAEDANS or IAF dyes at Cys^{374} according to the method of Miki and co-workers.

3.3 Co-sedimentation assays

To characterise the binding of formin to actin filaments, actin was polymerized overnight in the absence or presence of various mDia1-FH2 concentrations. Then the samples were centrifuged. Supernatants and pellets were applied separately to a SDS-polyacrylamide gel. The given protein band intensities were determined with a Syngene Bio-Imaging system.

3.4 Temperature-dependent Förster-type resonance energy transfer (FRET) experiments

To calculate the FRET efficiency the fluorescence intensities of the donor (IAEDANS) were recorded in the presence and absence of the acceptor (IAF). For the interpretation of the FRET results, the temperature dependence of the relative *f'* is presented. The larger temperature induced changes in the value of the normalized FRET efficiency are indicative for a more flexible protein matrix.

3.5 Steady-state anisotropy measurements

The IAEDANS-labelled actin containing samples were irradiated with plane-polarized light, and the degree of polarization of the emitted fluorescence was analysed. Those fluorophores can be excited that are aligned in the plane of the incident radiation and they are able to emit

fluorescence only. In the time interval between the absorption and the emission, the molecule can move out of the plane of polarization, so the emitted radiation will be depolarized to a degree that depends on the extent to which the molecule has moved. Small molecules and flexible protein matrices are moving faster and the degree of depolarization is higher than in the case of stiff, rigid protein structures.

3.6 Steady-state fluorescence quenching experiments

The steady-state fluorescence quenching data were first analysed by using the classical Stern-Volmer equation. The modified form of the classical Stern-Volmer relation (the Lehrer equation) was used when the samples contained more than one fluorophore population with different accessibilities.

3.7 Fluorescence lifetime-quenching measurements

All data were fitted to double exponential decay curves assuming a constant, frequency-independent error in both phase angle and modulation ratio. The goodness of the fit was determined from the value of the reduced χ^2. Average fluorescence lifetimes were calculated assuming discrete lifetime distributions.

3.8 Fluorescence lifetime and emission anisotropy decay measurements

Freshly prepared glycogen solution was used as a reference (lifetime = 0 ns). The fluorescence lifetimes of the fluorophore were determined by the use of nonlinear least-square analysis. Average fluorescence lifetimes (τ_{aver}) were determined from the results of the analysis assuming discrete lifetime distribution. The anisotropy is expected to decay as a sum of exponentials. The experimentally obtained data were fitted to a double exponential function.

4 New Results

1. The mDia1FH2 formin fragment induced structural rearrangement of actin filaments can be demonstrated by fluorescence emission anisotropy method and the time dependence of the alteration of the structure is traceable as well.

2. mDia1FH2 affects the conformation of the actin protomers at subdomain 1, depending on the applied ionic strength.

3. Tropomyosin stabilised the flexible formin-bound actin filaments while its affinity for the actin filaments did not change. The effect of tropomyosin on the actin filaments was independent of the concentration of KCl, but depended on the $MgCl_2$ concentration.

4. Heavy meromyosin stabilized the flexible structure of formin-bound actin filaments.

5 Discussion

5.1 The effect of mDia1-FH2 on the structure of actin studied by fluorescence quenching

First we wanted to explore the conformational changes in the actin filaments induced by formin binding. Steady-state and time-dependent fluorescence quenching proved to be the most informative methods for our investigations. We applied acrylamide as a neutral quencher and characterised the accessibility of the IAEDANS label attached covalently to Cys^{374} of actin protomers. In the absence of acrylamide the fluorescence emission intensity of IAEDANS was slightly lower in the presence of formin than in its absence suggesting that the binding of the FH2 domain changed the microenvironment of the fluorophore in the subdomain 1.

The analyses with the Lehrer equation showed increase in the value of K_{SV} when formin was added to the samples. At 500 nM mDia1-FH2 concentration the K_{SV} was approximately three times larger ($6.2 \pm 0.1\ M^{-1}$) than in the absence of formins, while the fraction of the quenchable fluorophores (α) decreased to 71 %.

To study the dependence of the observed effect the experiments were repeated at various formin concentrations and the data showed formin concentration dependence, i.e. the effect of formin depended on the formin : actin concentration ratio. The highest K_{SV} was observed at around 500 nM formin, above this concentration the value of K_{SV} decreased, and at 3 μM formin concentration the value of K_{SV} ($2.2 \pm 0.1\ M^{-1}$) was similar to the initial value we obtained in the absence of formins ($2.3 \pm 0.1\ M^{-1}$).

To further explore this effect we carried out the steady-state quenching experiments at higher ionic strength (50 mM KCl and 1 mM $MgCl_2$) as well, to test whether quenching was sensitive to the salt concentration. The effect of formin on the actin filaments depended on the ionic strength.

There is a good agreement between the K_{SV} values from steady-state and time-dependent quenching results, indicating that the contribution of the static quenching mechanisms to the overall quenching process is negligible.

5.2 Study of the formin-induced flexibility reduction using FRET

To describe the effects of skeletal muscle tropomyosin and heavy meromyosin on formin-bound actin filaments we applied steady-state and time dependent fluorescence methods. As full-length myosin precipitates at relatively low ionic strengths (i.e. under the experimental conditions applied here) we used myosin fragments for the investigations. The first technique we used was the same temperature dependent FRET method we applied previously to study the effect of formin on the flexibility of actin filaments. Using this method we were able to characterize the effect of tropomyosin and heavy meromyosin on the dynamic properties of the formin-bound actin filaments (*Fig. 1*).

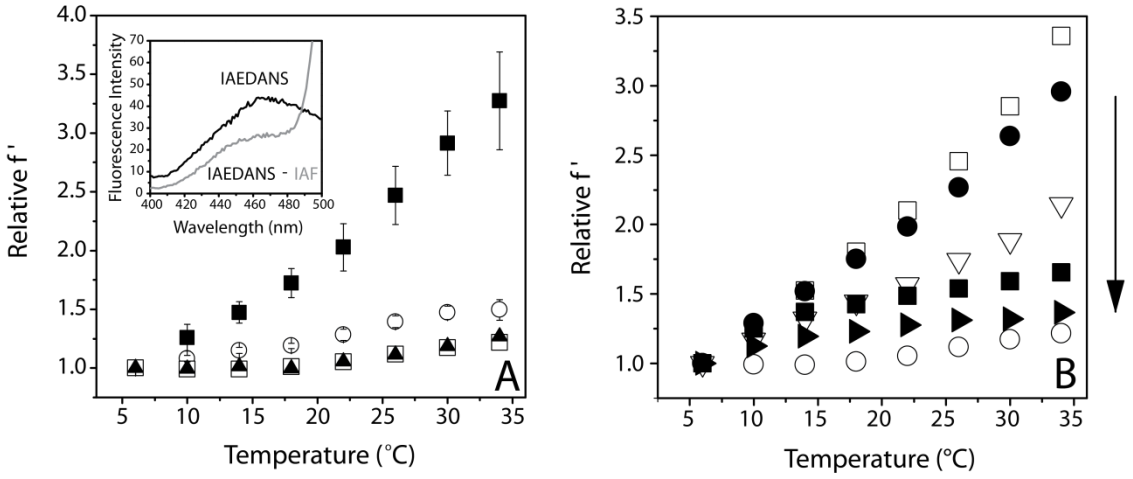

Figure 1 – Temperature dependence of the normalized FRET efficiency (relative *f'*, flexibility) for actin filaments. (**A**) Tropomyosin decreases the flexibility of formin-bound actin filaments. The experiments were carried out with 10 μM actin in the absence of actin-binding proteins (empty squares), or in the presence of mDia1-FH2 (500 nM; filled squares). The data obtained in the presence of tropomyosin (2 μM) and in the absence (filled triangles) or presence (empty circles) of 500 nM formin are also shown. The errors presented are standard errors from at least three independent experiments. The inset shows the fluorescence intensity of the donor measured in the absence of actin-binding proteins. The data were obtained in the absence (IAEDANS) or in the presence (IAEDANS-IAF) of the acceptor (as indicated). (**B**) Heavy meromyosin decreases the flexibility of formin-bound actin filaments. The experiments were carried out with 5 μM actin in the absence of actin-binding proteins (empty circles). Empty squares represent the data with 500 nM mDia1-FH2 in the absence of HMM. The data obtained in the presence of 500 nM formin and 1 μM (filled circles), 3 μM (empty triangles), 5 μM (filled squares) or 10 μM (filled triangles) skeletal muscle heavy meromyosin are also shown. Arrow in the right indicates the increase of the HMM concentration.

5.3 Study of the formin-induced flexibility reduction using anisotropy decay

The next method we used to confirm our previous FRET results was the measurement of anisotropy decay. This method was used to describe the dynamic properties of actin many times before. Our experiments were carried out with IAEDANS-labelled actin filaments. The evaluation of the data resulted in two rotational correlation times. The value of the shorter rotational correlation time was between 1-4 ns, and showed no formin, tropomyosin or heavy meromyosin concentration dependence. The value of the longer rotational correlation time showed formin-concentration dependence and its value was ~ 700-900 ns in the absence of formin which decreased to ~ 225-250 ns in the presence of 500 nM mDia1-FH2 (*Fig. 2* and *3*). This observation is in agreement with our previous results and indicated that formin binding made the actin filaments more flexible. To test the effect of tropomyosin on the flexibility of formin-bound actin filaments anisotropy decay experiments were performed in the presence of TM at various concentrations (*Fig. 2*).

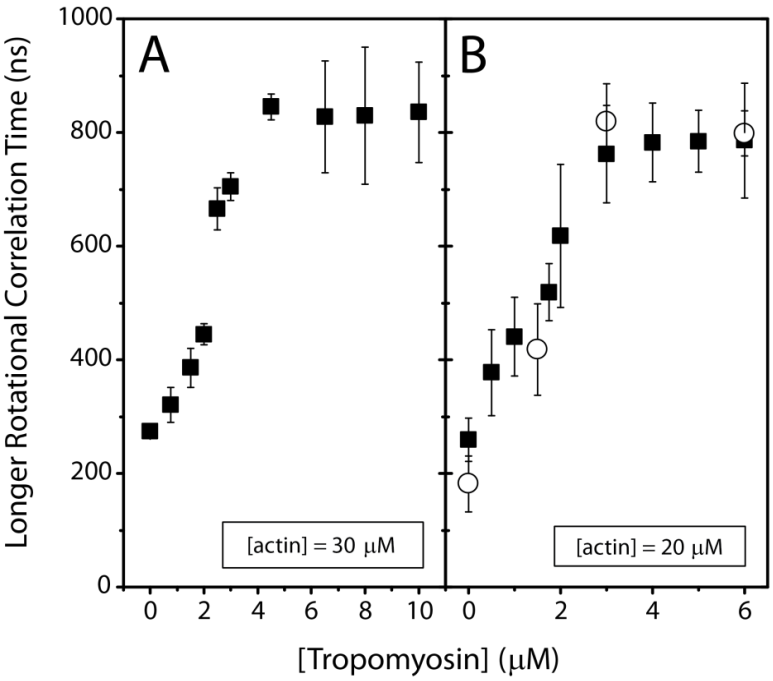

Figure 2 – Tropomyosin affects the anisotropy decay of formin-bound IAEDANS-actin filaments. The figure shows the tropomyosin concentration dependence of the longer rotational correlation times obtained for actin filaments. In panel **A** the actin concentration was 30 μM and the sample also contained 1.25 μM mDia1-FH2. Panel **B** shows the tropomyosin concentration dependence of the longer rotational correlation time measured with 20 μM actin and either 500 nM mDia1-FH2 (filled squares) or 500 nM mDia1-FH1FH2 (empty circles). The errors presented are standard errors from at least three independent experiments.

Co-sedimentation experiments were carried out to exclude the possibility that the binding of formin weakens the affinity of tropomyosin for actin. The amount of tropomyosin in the pellets was independent of the presence of formin, indicating that the mDia1-FH2 did not modify substantially the affinity of Tm for actin.

Since previous studies showed that the effect of formins on actin filaments depends on ionic strength, we tested the effect of potassium and magnesium on the interaction of Tm and mDia1-FH2-bound actin filaments by using fluorescence anisotropy decay. The interaction between Tm and actin is magnesium dependent. The affinity of tropomyosin for actin, and the corresponding association and dissociation rates were magnesium concentration dependent. The affinity is higher at higher $MgCl_2$ concentrations (K_D = 2.4 μM at 0.5 mM $MgCl_2$ and K_D = 0.5 μM at 2.5 mM $MgCl_2$).

We repeated the anisotropy decay experiments with formin-bound actin filaments in the presence of HMM at various concentrations (*Fig. 3*).

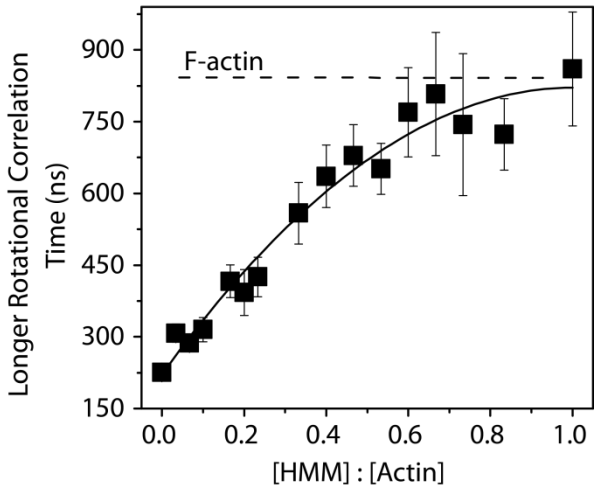

Figure 3 – Heavy meromyosin affects the anisotropy decay of formin-bound IAEDANS-actin filaments. Dashed line indicates the value of the rotational correlation time measured in the absence of formin and HMM. The actin and mDia1-FH2 concentrations were 30 μM and 1.25 μM, respectively. The errors presented are standard errors from at least three independent experiments.

The value of the longer rotational correlation time increased with increasing TM or HMM concentrations. This indicates that formin-bound actin filaments were stiffer in the TM or HMM-bound state.

5.4 Study of the formin-induced flexibility reduction using steady-state anisotropy

Steady-state fluorescence anisotropy measurements confirmed our previous results. The evaluation of the steady-state anisotropy data provides information on the flexibility of the experimental material in the cuvette, where a higher anisotropy value indicates a stiffer protein matrix. These experiments were also carried out with IAEDANS-labelled actin filaments.

Fluorescence anisotropy values of actin filaments alone were 0.25-0.26. After an overnight incubation with 500 nM mDia1-FH2 (that was the applied formin concentration in each sample), the value of anisotropy decreased to 0.14-0.17 depending on the time of incubation. This observation is in correlation with our previous results obtained with other methods and confirms that the flexibility of the protein matrix increased upon binding of formin.

When tropomyosin was added to the formin-bound, flexible actin filaments in different concentrations, the anisotropy started to increase slowly and a plateau appeared after ~ 30 minutes at anisotropy values ~ 0.25.

When HMM was added to the sample after the overnight incubation with formin, the anisotropy increased rapidly (plateau was reached ~ 1 minute after the addition of HMM) to the values which are typical of actin alone (0.24-0.26 or above – depending on the concentration of the given HMM).

6 Summary

Fluorescence quenching experiments proved that the accessibility of the quencher molecules is much higher to the protomers of formin-bound actin filaments. The effect of formin affected the rate of quenching, which shows that the protein matrix around the Cys^{374} has been changed, thus mDia1 formin changed the conformation of the subdomain 1 of actin protomers. These intramolecular alterations undoubtedly show that the flexibility of the subdomain 1 of the actin protomers became flexible and confirm the fact that formins regulate the flexibility of actin filaments through long range allosteric interactions.

Temperature-dependent FRET, fluorescence emission anisotropy and fluorescence anisotropy decay methods showed that subsequent binding of heavy meromyosin stiffen the structure of the formin-bound actin filaments and restored the dynamic state of actin. Complete stabilisation appears to occur at substoichiometric HMM concentrations.

According to our results the binding of tropomyosin stabilised the flexible formin-bound actin filaments. The effect of tropomyosin on the actin filaments was independent of the concentration of KCl, but depended on the $MgCl_2$ concentration.

7 Conclusions

These new results emphasize the importance of the intramolecular conformational changes induced by formins in the structure of actin filaments. The mDia1 formin changed the conformation of the protein matrix in the subdomain 1 of the actin protomers. It seems reasonable to assume that these flexible actin filaments serve well-defined biological aims, which are manifested under special intracellular conditions. However, one would also assume that other conditions require actin networks with mechanically stabile, more rigid filaments. We therefore expected that actin-binding proteins could reverse the formin-induced structural changes by binding to filaments. Tropomyosin and heavy meromyosin were tested whether they can stabilize the conformation of the actin filaments. The results presented in my thesis indicate that both of these abundant actin-binding proteins were able to reverse the formin induced conformational changes. The experimental data proved that the molecular mechanisms exerted by the effect of tropomyosin and heavy meromyosin play a central role in the molecular mechanisms regulating the conformational dynamics of actin filaments. We speculate that the greater flexibility of the filaments observed in the presence of formins may result from the rapid and special way of formin-assisted polymerization. For the proper functioning of actin filaments, the conformational state with a more rigid structure is probably preferable, and thus there are molecular repair mechanisms (with the binding of actin-binding proteins) which reverse the formin-induced flexibility changes. With the results presented here there are now two abundant binding partners of actin identified – Tm and HMM – that can play such regulatory roles. Their common property is that the binding of both of these proteins cooperatively affects the structure of the actin filaments along a stretch of several protomers.

My present results serve new perspectives towards further investigations to identify other actin-binding proteins which play a regulatory role in the fine-tuning of actin conformation. These proteins should be studied in complex systems to reveal how the different effects of actin-binding proteins on the conformational properties of actin are superimposed (synergistically or antagonistically) to establish the overall conformational dynamics and the biological function of cellular actin structures. This way we can examine the different contender processes during the organization and realignment of the cytoskeleton.

8 Publications

8.1 Publications related to the Thesis

1. **The Effects of Formins on the Conformation of Subdomain 1 in Actin Filaments**. **Ujfalusi, Z.**, Barkó, Sz., Hild, G., Nyitrai, M. *Journal of Photochemistry and Photobiology B-Biology* **98:(1)** pp. 7-11. (2010)

   IF: 1.871; Citations: 2

2. **The effect of tropomyosin on formin-bound actin filaments**. **Ujfalusi, Z.**, Vig, A., Hild, G., Nyitrai, M. *Biophysical Journal* **96:(1)** pp. 162-168. (2009)

   IF: 4.390; Citations: 2

8.2 Conference presentations related to the Thesis

1. **Aktin-kötő fehérjék hatása az aktin filamentumok flexibilitására. Ujfalusi, Z.**, Vig, A., Nagy, N., Kovács, M., Hild, G. and Nyitrai, M., – oral presentation at the XXIIIrd Congress of the Hungarian Biophysical Society (23-26 August, 2009, Pécs).

2. **The effect of actin binding proteins on the flexibility of formin-bound actin filaments**. **Ujfalusi Z.**, Vig, A., Nagy, N., Kovács, M., Hild, G. and Nyitrai, M. – oral presentation at the Symposium for Graduate Students in Biology (12-13 November, 2009, Pécs).

3. **The effect tropomyosin and heavy meromyosin on the flexibility of formin-bound actin filaments. Ujfalusi, Z.**, Nyitrai, M. and Hild, G. – oral presentation at the Intracellular Fluorescence Spectroscopy Satellite Conference of the 8th European Biophysics Congress (20-22 August, 2011, Pécs).

8.3 Other publications

1. **Conformational Changes in Actin Filaments Induced by Formin Binding to the Barbed End**. Papp, G., Bugyi, B., **Ujfalusi, Z.**, Barkó, Sz., Hild, G., Somogyi, B., Nyitrai, M. *Biophysical Journal* **91:(7)** pp. 2564-2572. (2006)

   IF: 4.757; Citations: 6

2. **The effect of pyrene labelling on the thermal stability of actin filaments**. Halasi, Sz., Papp, G., Bugyi, B., Barkó, Sz., Orbán, J., **Ujfalusi, Z.**, Visegrády, B. *Thermochimica Acta* **(445)** pp. 185-189. (2006)

   IF: 1.417; Citations: 2

3. **The effect of pH on the thermal stability of alpha-actin isoforms**. Papp, G., Bugyi, B., **Ujfalusi, Z.**, Halasi, Sz., Orbán, J. *Thermochimica Acta* **(82)** pp. 281-285. (2005)

   IF: 1.230; Citations: 4

Cumulative impact factor: 13.665 Sum of independent citations: 16