Thermodynamic and spectroscopic analysis of actin isoforms prepared from different muscle tissues

PhD thesis booklet

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I. INTRODUCTION

Actin is a highly conservative protein occurring in high abundance in all eucaryotes. Its occurrence in many different cells emphasizes its central role in the function of living systems. It is one of the building subunits of the thin filament in the muscle tissues. Apart from its essential role in the muscle contraction it can also be found as a part of the cytoskeleton, as it is the major component of the microfilament system. The cytoskeleton has central role in the life of the living eukaryotic cells as it is involved in the intracellular transport of molecules, the endo- and exocytosis and cell movements as well.

First it was prepared from skeletal muscle by Bruno F. Straub and Albert Szentgyörgyi (*1*). They realised that a special protein can be separated from the so called myosin solution prepared according to Kühne's and Engelhardt's method. They found that this protein can activate the ATPase activity of myosin and together they create a more viscous solution at high salt concentrations, which is due to their specific interaction. Feuer and Straub demonstrated that actin molecules hydrolise ATP during the so called polymerisation process (*1* ,*2*).

According to electron microscopic and X-ray diffraction experiments scientists created models of actin forming a filament of helical structure. Until nowadays the most used and generally spread model was based on a crystallographic study of actin monomer in complex with *DNase I* at a resolution of 2.8 Å (in 1990) (*3*).

Today the actin's complex with myosin (acto-myosin complex) is the most frequent target of investigations, more recently its complex with the actin binding proteins has come into the forefront of scientific investigations. Beside this, we should not forget the importance of the different isoforms of actins. A lot of information is still hidden in the structural and physiological differences of isoforms that have influences on their role in the cell.

Myosin as actin binding molecule

We studied the interaction of myosin II (I will refer to as myosin) from the wide family of the so called myosin motor proteins. This molecule can be found in eukaryotic animal cells. In multicellular organisms the myosin is a highly important element of muscle contraction beside the actin (*4*). Muscle contraction and other processes of cell motility are based on the cyclic interaction of the head portion of myosin with actin.

Myosin is an asymmetric molecule due to its long helical tail and the more compact, globular heads. The tails let myosin molecules bundle and create the thin filament and the heads

contain the *actin*- and *ATP-binding regions*. Myosin subfragment 1 (myosin S1) is a digestive subfragment of the myosin that was first used for structure deteremination by X-ray crystallography (*5*). This subfragment as a water soluble, molecular mass of ~120 kDa protein was used in our experiments because this is the smallest fragment of actin capable of both actin binding and ATPase activity (*6*, *7*).

The acto-myosin duty cycle

The bound and disconnected states of actin and myosin create a mechanic and an energetic cycle. During the cycle the myosin bound ATP is hydrolysed to ADP through an intermediate complex of ADP·P_i. When ATP is bound to myosin actin binding is blocked and only when ADP·P_i state is reached the acto-myosin complex can be created. The released energy is converted to a structure change of the so called neck region which transfers the complex to a strongly bound state at the same time. In the next steps the inorganic phosphate (P_i) and later the ADP dissociates from the myosin head and the complex remains in the strong binding state. In the previous step, due to the conformational change, the myosin creates a pulling force and slides along the actin filament. A newly bound ATP can end the strongly binding state of the complex and therefore the myosin detach from the actin filament.

Actin

Actin isoforms in the nature show high similarity in their highly conservated amino acid sequences. Actin plays important role in cellular transport, cellular motility, endo- and exocitosys and cytokinesys as well. Actin is the basic element of microfilament system that is one of the three filamentous systems in cells beside microtubules and intermedier filaments.

In cells it can be found as a monomer (globular or G-actin) or polymer (filamentous or F-actin) in complex with a divalent cation and a nucleotide in its central cleft between its large and small domain that can be divided into 2-2 subdomains. This cation is assumed to be magnesium in the physiological form of the protein. The monomer actin has a molecular mass of 43 kDa.

Both actin monomers and the polymerised actin filaments are sensitive to the physico-chemical properties of their environment. The molecular structure differs depending on the bound nucleotide: with ATP it forms a closed structure and binding other nucleotides (e.g. ADP) the molecule transforms to an opened state meaning wider cleft between the main domains.

Due to the polymerisation process actin forms a homopolymer which is a double helix with a

width of 12 nm (*12*). This spiral structure can resolved as a steep left-handed one stranded filament and as two right-handed strands twisting around each other (*13*).

Actin isoforms

In the actin family more than 100 unique sequences exist and their spatial structure and function show high similarity. The same isoforms are highly conserved at different developmental levels of animals (*14*). Six isoforms of actin can be distinguished in the nature based on their isoelectric points (three α-isoforms: pI=5.40; one β-isoform: pI=5.42; two γ-isoforms: pI=5.44). The β- and γ-isoforms can be found mainly in non-muscle cells. The α-isoforms are found mainly in the muscle tissues and can differ only in a few of their amino acid residues. It is hypothesised that each of the muscle isoactin is specially adapted to the function of its respective tissue and the minor variations among them have developmental and/or physiological relevance.

The most studied actin isoform is the α-skeletal isoform. It differs only by 4 amino acids from the α-cardiac isoform (*11, 15, 16*). In contrast to the small differences they are involved in different physiological processes that is represented by the different function of the heart muscle and the skeletal muscle cells (*17-20*).

Aims

The final aim of my experiments was to get to know the dynamics of actin molecule, and the interaction with moysin in details. We studied the acto-myosin complex using actins and myoisin prepared from heart and skeletal muscle tissues to reveal tissue specific differences related to the isoforms of the molecules and find homology based similarities as well.
The thermodynamic, polymerisation and acto-myosin complex creating properties of the actin isoforms were investigated under different circumstances, such as:

1. *Effect of the ionic strength, and the effect of the divalent cations (Ca^{2+} / Mg^{2+}).*
2. *Effect of pH.*

   One of the known and most studied conditions of the microenvironment in the case of proteins.
3. *Effect of nucleotides and nucleotide analogues.*

   Actin has ATPase activity. The dynamic and structural changes due to the ATP hydrolysation are not understood in details and its exact role is also not clear. The aim of our study was to investigate the intermedier ADP·P_i state of the ATPase cycle by using mimicking nucleotide analogues (BeF_x- and AlF_4·ADP).

We studied acto-myosin complex to reveal kinetic parameters describing the molecular interactions of actin and myosin, using different isoforms or more precisely molecules extracted from heart and from skeletal muscle:

4. *Determination of acto-myosin complex dynamics by appropriate kinetic parameters (by stopped flow analysing technique).*
5. *Monitoring of the ATPase activity of myosin S1, and the activating effect of actin filaments on the ATPase activity of subfragment 1.*

It was also our aim to compare and to understand the effect of toxins on the structure of actin filament:

6. *Separate and unique effect of toxins on actin filament.*

   The toxins – in spite of that they creat artificial state – helps us to discover the structure and function of actin filament.
7. *Collective effect of toxins and nucleotide analogues.*

   As supplemental information we planned to study and compare the separate and collective effect of toxins and nucleotide analogues on the structure of the actin filament through its thermodynamic properties.

II. APPLIED TECHNIQUES

Actin preparation from muscle tissue(s)

Both the cardiac and skeletal actins were prepared from acetone-dried muscle powder prepared by the method of Feuer et al. (*1*). The method of Spudich and Watt (*22*) was followed to prepare the actin isoforms from bovine heart (cardiac α-isoform) and rabbit skeletal muscle (skeletal α-isoform). Both isoforms are well studied isoforms of actin and are generally prepared from bovine heart and rabbit skeletal muscle. Their amino acid sequences totally match with the same human isoforms.

Further important preparation methods and steps

- *Exchange of divalent ions*

The bound calcium was replaced with magnesium on the actin monomers before polymerisation by the method of Strzelecka and colleagues – when it was necessary.

- *Preparation of ADP-actin*

The actin monomer bound ATP was changed for ADP by the method of Drewes and Faulstich (*23*).

- *Preparation of pyrene labelled G-actin*

For fluorescence based measurements the pyrene-iodoacetamide (pyrene) labelled F-actin was prepared by incubating actin filaments with pyrene dissolved in DMF at a concentration of 5 mg/ml for the stack solution. After the incubation the labelled actin was purified in several steps (dyalisation, centrifugation). The fluorofor binds the actin at Cys^{374} amino acid.

III. MEASUREMENT TECHNIQUES

The processes where proteins are included follows well defined laws and these processes or the laws may be discovered using the appropriate techniques. We applied the following techniques and methods:

- *Spectrophotometry*

Spectroscopic methods were used to determine protein concentration, fluorofor labelling efficiency, and to measure pyrene labelled actin's excitation and emission spectra. The *Coupled ATPase activity test* and the polymerisation test require this method as well. „Stopped flow" technique also utilises spectroscopy to follow molecular interactions and to reveal kinetic parameters of the interaction.

- *„Stopped flow" technique*

This is one of the most widely used fast-kinetic measurement techniques. To determine the dynamic parameters of actin we studied the change of pyrene labelled F-actin.

- *Differencial scanning calorimetry (DSC)*

The differential scanning calorimetry is a powerful biophysical tool to characterise the thermal properties of proteins at molecular level and sometimes resolves information at submolecular level as well (*24*). It is widely used in the measurements of the calorimetric features of the muscle actin and its associates as well. The measured thermal parameters can inform us about the thermodynamic properties of a protein.

- *Cooperativity in the actin filament*

Cooperativity was demontrated in experiments where actin filament was treated by phalloidin, berillium-fluoride, myosin or with some regulation proteins. We applied the model described recently (*25*) to analyze the phalloidin concentration dependence of the DSC data obtained with actin filaments. The model assumes that phalloidin can stabilise not only the conformation of the protomer it is bound to, but in the case of cooperative binding, the toxin can also stabilise adjacent actin protomers along the actin filament.

IV. RESULTS and DISCUSSION

Based on the experimental results the followings can be stated:

ISOFORMS:

Our results can demonstrate that the few differences between the amino acid sequences of the α-actin isoforms have an influence on the thermal properties and on polymerisation dynamics of the isoforms. Based on the presented experimental data the significance of small difference in amino acid sequence might alter proteins' physico-chemical properties and those demonstrably appear in the work of different muscle tissues. Summarizing the differences:

Polymerisation dynamics of heart muscle originated actin is more sensitive to ionic strength of the environment compared to the actin prepared from skeletal muscle. Under similar circumstances (cation concentration, pH, nucleotides, ionic strength) cardiac isoform polymerises always slower then the skeletal isoform.

Using **calorimetric technique** (DSC) we confirmed results of other techniques denoted as the α-skeletal isoform of actin is more stable compared to the α-cardiac isoform, especially in the presence of Ca^{2+} ions. The cardiac isoform shows more sensitivity toward the change of the environment considering the thermodynamic parameters as well.

1. Compared to other chemical agents the cations do not change greatly the actin's thermodynamic stability. The divalent cations (Mg^{2+}, Ca^{2+}) define the lifetime of the actin molecule but independently of which is bound to the filament the structure is the same. When we compare the isoforms, the Ca^{2+}-skeletal isoform and the Mg^{2+}-cardiac isoform is slightly more stable compared to the isoform binding the other cation. The monovalent cations have less stabilisation effect with increasing concentration. They can only affect the polymerisation speed (critical concentration decreases) but not the thermodynamic properties.
2. A small difference can be shown in the thermodynamic properties of actin isoforms due to a different ambient pH level. Both isoforms are more stable against heat close to the physiological pH in muscle cells (pH 7.0) compared to lower and higher pH.
3. Significant differences were presented in the ADP-binding form of α-cardiac and α-skeletal actin filaments. It was demonstrated that both ATP- and ADP binding forms of skeletal isoform are more stable compared to the cardiac one, based on thermodynamic parameters. In both cases the width (FWHM) of the DSC peaks corresponding to the ADP-F-actin forms are bigger then at the case of ATP-binding filaments. This indicates bigger intremolecular cooperation in ATP-binding actin filaments. On the denaturation curve of the heart-originated actin filaments the peaks of ADP-skeletal and ADP-cardiac isoform actin subpopulation are clearly separable. We can declare that the nucleotides have a high impact on the determination of the

physiological role of actin because the different stability of filaments would lead to different work and behaviour in cellular processes and in muscle contraction.

Kinetic parameters of acto-myosin complex:

4. The kinetic parameters of the acto-myosin complex show a difference in the case of the isoforms. The affinity values (K_A , K_{AD} and K_{DA}) represent important information of the actin-myosin interaction. Other examined parameters of skeletal muscle myosin-S1 and actin filaments do not differ significantly. The skeletal actin isoform filaments presented bigger affinity compared to cardiac isoform filaments which we interpret as the skeletal isoform creates more effective complex with myosin-S1.
5. Based on the actin activated S1 ATPase activity the actin and myosin showed greater activating effect when the molecules were prepared from the same tissue. Compared to it the activation effect of actin prepared from another tissue then the myoisin was less. In all of the experiments skeletal isoform filaments showed greater activation in contrast with the cardiac isoform small activation. This is partly due to the greater affinity of skeletal actin to S1, presented in the previous paragraph.

Concluding the above written facts we declare that the cardiac α-actin isoform has slower polymerisation dynamics in both nucleotide binding state and the formed filament is always thermodynamically less stable under the studied parameters of environment, furthermore it creates the acto-myosin complex at a lower affinity compared to the α-skeletal actin isoform.

EFFECT of TOXINS and NUCLEOTIDE ANALOGUES

6. According to previous measurements phalloidin and jasplakinolide presented similar stabilisation effect on skeletal actin filaments. The increase of resistance against heat denaturation can be justified based on thermodynamic parameters. These results are only a base of comparison for further nucleotide state measurements.
7. We demonstrated that phalloidin and the nucleotide analogues (BeF_x / AlF_4·ADP) use different mechanisms to stabilize the structure of actin filaments.
   - The stabilisation effect of phalloidin is no more cooperative in the case of nucleotide analogue-filled F-actin. The ADP·Pi state formed by the analogues does not allow to spread the effect of bound toxins to the neighbouring protomers in the filament.
   - Using jasplakinolide and nucleotide analogue – in contrast to the case of phalloidin and analogue – additive stabilisation effect of chemical agents was not demonstrable. The nucleotide analogue effect incapacitate the toxin to further stabilise the filament. Probably the analogue decreases the affinity of the toxin to the actin filament by altering its structure.
   - Jasplakinolide and phalloidin have different behaviours beside and despite the numerous similar properties.

V. BIOLOGICAL RELEVANCE

The accumulated data suggest that the stability of the actin filament plays a role in the function of the actin cytoskeleton. For example, the altered stability of the protein matrix in the case of the ADP-actin filaments, and thus their different conformation may explain their altered affinity to the actin binding proteins as well.

It is hypothesised that each of the muscle isoform of actin is specially adapted to the function of its respective tissue and the minor variations among them have developmental and/or physiological relevance. Taking into account that in the heart the ratio of skeletal and cardiac isoform is different from the ratio of isoforms in skeletal muscle tissue we might conclude that each specific work requires specific isoform. When the ratio in the heart changes due to increased mechanical stress or by pathological reasons then the reason of the change is to adapt for the new circumstances. The demonstrated thermodynamic and dynamic differences are the consequences of structural differences required for appropriate work in the different tissues.

Nucleotides have an important role in defining the structure of the monomer and the actin filament as well. It was demonstrated for several *actin binding molecules* that those bind differently to the actin depending on the bound nucleotide. These molecules might have an influence on the nucleotide exchange rate as well. Through this regulations of affinity and nucleotide release these molecules define the rate of dynamic renovation, the structure and based on these effects finally determine the functionality of actin in cells.

Toxins stabilise cooperatively the structure of ADP-actin filaments (prepared from ATP-actin monomers). When F-actin is treated by toxins and nucleotide analogues the separately demonstrated cooperative stabilisation of chemical agents disappears or at least the additive stabilisation effect does not occur. The role of cooperativity may be that with small energy investment (in presence of few ATP) large compartment of the filament can change the structure, minimising the energy required to keep actin in its functional form. The disappearence of cooperativity in the case of ADP·P_i state might be due to the highly stable ATP/ADP·P_i structure of F-actin, which makes the further cooperative changes along the filament impossible.

Based on our results it is highly important to study the acto-myosin complex. Biologically the role of the isoforms, and from the viewpoint of physics the parameters of force creation and acto-myosin cycle is waiting for further investigations to create a good basis to understand the complex regulation of muscle contraction.

VI. ARTICLES

The publications the thesis is based on:

THERMODYNAMIC CHARACTERIZATION OF DIFFERENT ACTIN ISOFORMS; József Orbán, Szulamit Halasi, Gábor Papp, Szilvia Barkó and Beáta Bugyi; Journal of Thermal Analysis and Calorimetry, Vol. **82** (2005) pp. 287–290, IF: 1,425

THE EFFECT OF pH ON THE THERMAL STABILITY OF α-ACTIN ISOFORMS; Gábor Papp, Beáta Bugyi, Zoltán Ujfalusi, Szulamit Halasi and József Orbán; Journal of Thermal Analysis and Calorimetry, Vol. **82** (2005) pp. 281–285, IF: 1,425

THERMAL CHARACTERISATION OF ACTIN FILAMENTS PREPARED FROM ADP-ACTIN MONOMERS; József Orbán, Kinga Pozsonyi, Krisztina Szarka**,** Szilvia Barkó, Emőke Bódis and Dénes Lőrinczy; Journal of Thermal Analysis and Calorimetry, Vol. **84** (2006) 3, pp. 619-623, IF: 1,438

THE EFFECT OF JASPLAKINOLIDE ON THE THERMODYNAMIC PROPERTIES OF ADP·BeF_X BOUND ACTIN FILAMENTS; Roland Kardos, Andrea Vig, József Orbán, Gábor Hild, Miklós Nyitrai, Dénes Lőrinczy; Thermochimica Acta, Vol. **463** (2007), pp. 77-80, IF: 1,562 (2007-es)

NUCLEOTIDE DEPENDENT DIFFERENCES BETWEEN THE α-SKELETAL AND α-CARDIAC ACTIN ISOFORMS; József Orbán, Dénes Lőrinczy, Miklós Nyitrai, Gábor Hild; BBRC, Vol. **368** (2008), pp. 696-702, IF: 2,855 (2006-os)

NON-COOPERATIVE STABILIZATION EFFECT OF PHALLOIDIN ON ADP.BeF_X- AND ADP.AlF_4-ACTIN FILAMENTS; József Orbán, Dénes Lőrinczy, Gábor Hild and Miklós Nyitrai; Biochemistry, Vol. **47** (2008), pp. 4530-4534, IF: 3,368 (2007-es)

Not included publication:

THE EFFECT OF PYRENE LABELLING ON THE THERMAL STABILITY OF ACTIN FILAMENTS; Szulamit Halasi, Gábor Papp, Beáta Bugyi, Szilvia Barkó, József Orbán, Zoltán Ujfalusi, Balázs Visegrády; Thermochimica Acta, Vol. **445** (2006), 185-189, IF: 1,417

Cummulative impact factor of publications: 13,49

Abstracts used in preparation of thesis:

József Orbán, Szulamit Halasi, Gábor Papp, Szilvia Barkó and Beáta Bugyi - **Thermodynamic caracterization of different alpha-actin isoforms.** 16. Ulm-Freiberger Kalorimetrietage (2005, Freiberg, Németország)

Papp Gábor, Bugyi Beáta, Ujfalusi Zoltán, Halasi Szulamit és *Orbán József* - **The effect of pH on the thermal stability of alpha–actin isoforms.** 16. Ulm-Freiberger Kalorimetrietage (2005. március, Freiberg, Németország)

Halasi Szulamit, Papp Gábor, Bugyi Beáta, Barkó Szilvia, *Orbán József*, Ujfalusi Zoltán és Visegrády Balázs – **The Effect of Pyrene Labelling on the Thermal Stability of Actin Filaments.** 16. Ulm-Freiberger Kalorimetrietage (2005. március, Freiberg, Németország)

Beáta Bugyi, Gábor Papp, *József Orbán*, Szulamit Halasi and Balázs Visegrády - **The effect of toxins on the thermal stability of actin filaments as revealed by differential scanning calorimetry.** 16. Ulm-Freiberger Kalorimetrietage (2005, Freiberg, Németország)

József Orbán, Miklós Nyitrai, Katalin Ajtai, Béla Somogyi, Gábor Hild - **Spectroscopic and functional differences between actin isoforms.** FEBS Special Meeting on 'Cytoskeletal dynamics: from cell biology to development and disease (Helsinki, Finnország, 2005. 06. 12-16.)

Roland Kardos, Andrea Vig, *József Orbán*, Gábor Hild, Miklós Nyitrai and Dénes Lőrinczy - **The Effect of Jasplakinolide on the Thermodynamic Properties of ADP-BeF_x Bound Actin Filaments.** 17. Ulm-Freiberger Kalorimetrietage (2007, Freiberg, Németország)

József Orbán, Andrea Vig, Roland Kardos, Béla Somogyi, Gábor Hild, Dénes Lőrinczy and Miklós Nyitrai - **The Effect of Phalloidin on the Thermal Properties of the Skeletal ADP.BeF_x-F-actin.** Time and Space Resolved Methods in Molecular Biophysics Conference (Hünfeld, Németország, 2007. 05. 17-20.)

József Orbán, Andrea Vig, Roland Kardos, Béla Somogyi, Gábor Hild, Miklós Nyitrai and Dénes Lőrinczy - **Thermodynamic Characterization of BeF_x.ADP-F-Actin in the Presence of Different Cytotoxins as Revealed by Differential Scanning Calorimetry.** Regional Biophysical Conference (Balatonfüred, 2007. augusztus)

Andrea Vig, Réka Dudás, Tünde Kupi, *József Orbán*, Gábor Hild, Dénes Lőrinczy and Miklós Nyitrai - **The Effect of *Phalloidin* on cardiac ADP- Actin Filaments.** XV. International Conference on Biological Calorimetry (Pécs, 2008 május 24-30.)

Réka Dudás, Tünde Kupi, Andrea Vig, *József Orbán*, Miklós Nyitrai, Dénes Lőrinczy and Gábor Hild - **The effect of phalloidin on skeletal muscle ADP-actin filaments.** XV. International Conference on Biological Calorimetry (Pécs, 2008 május 24-30.)

Tünde Kupi, Réka Dudás, Andrea Vig, *József Orbán*, Miklós Nyitrai, Gábor Hild and Dénes Lőrinczy - **Effect of AMP PNP as a Nucleotide Analogue on Actin Filaments.** XV. International Conference on Biological Calorimetry (Pécs, 2008 május 24-30.)

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