Ph.D. THESIS

The interaction of actin and formin: EPR and DSC studies

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1 Introduction

Actin is one of the main component of the Eukaryotic cytoskeleton. In cellular structures actin has diverse functions involving the maintenance of cellular shape, movement of cells, intracellular transport processes, cell division, endo- and exocytosis, and many other cellular functions. In living cells globular actin monomers (G-actin) and filamental actin polymers (F-actin) can be found. Actin monomer is a 42.3 kDa protein consisting of two main domains. The domains are further divided into two subdomains (S1-4). The common binding motif, a hydrophobic cleft is formed between actin subdomain 1 and 3, that serves as a binding site for cations (Mg^{2+}, Ca^{2+}) and nucleotides (ATP, ADP, the ADP-P_i). Polymerisation of actin monomers result in actin filaments, which usually are described as a two-start, right-handed helix. The formation of filaments starts with a slow phase, the so called nucleation, when the G-actin molecules are linked to dimers and trimers (called nuclei). During the next phase, called elongation actin monomers binding becomes faster, the length of filament increases. Whereas the orientations of the linked monomers are the same, the filament has a polar structure. The last phase is a dynamic equilibrium, a so-called treadmilling mechanism, when the length of the filaments does not change. In this case, at both ends of the filaments monomer dissociation and association takes place as well, but at the positive end (barbed end) the association, at the negative end (pointed end) the dissociation process dominates.

The dynamics of the actin cytoskeleton - its rapid assembly and disassembly, which is essential for many cellular functions - is regulated *in vivo* by various actin-binding proteins (ABPs). Proteins controlling the nucleation process can be divided into three protein families. ARP 2/3 proteins form branched actin filaments. WH2 domain containing proteins help the formation of actin nuclei from pools of monomers. This family includes the Spire, Cordon-bleu (Coble), VopF / VopL and Leiomodin (Lmod) proteins. A third family of nucleation factors are formins that support the formation of unbranched actin filaments. Previous results support the view that formin binds to the hydrophobic cleft of actin, which is formed between the subdomain 1 and 3.

The formins are large, more than a thousand-amino acids containing proteins, which contain evolutionarily conserved domains. The formins are involved in cell motility, cell

polarity shaping, cell division, and in many other processes. Common structural features of the protein family are the formin homology (FH) domains. The proline-rich FH1domain occurs in most of formins, the highly conserved FH2 in all formins. The phylogenetic analysis of the FH2 domain showed seven subfamilies in animals (metazoan): Dia (diaphanous) DAAM (dishevelled-associated activator of morphogenesis), FRL (formin-related gene in leukocytes), FHOD (formin homology domain containing protein), INF (inverted formin), FMN (formin) and delphilin.

FH2 domain is an approx. 400-amino acid sequence, which is responsible for actin binding. Mammalian FRL1, mDia1 mDia2 FH2 domains and also the dimer FH2 domain comprises a mobile linker region near the N-terminus, which plays an essential role in the formation of dimers. The crescent-shaped formin monomers build up dimers, while linked together antiparallel. The profilin-binding domain of FH1 can be found in almost all formins. It is in the N-terminal near the FH2 domain and its length is highly variable (from 15 to 229 amino acids).

The parts of formins outside the FH1 and FH2 domains vary significantly. The third formin homology domain (FH3) in several formins is situated in the N-terminal direction next to the FH1 domain. Its role as an independent domain is still in question. In mDia1 formins the FH3 equivalent region comprises at least two distinct domains: the section DID (diaphanous inhibitory domain) and the one responsible for dimer forming.

The regulation of the mammalian Diaphanous- related formins (DRF) is the best known among all the formin families. DRFs include Dia, FRL and DAAM subfamilies, which are related based on intramolecular regulation similarities. In all the three subfamilies a Rho-GTPase-binding domain (RBD) can be found in the N-terminal region. In mDia1 formins a 241-amino acid auto-inhibitory region is located in the RBD, which is also referred to as diaphanous inhibitory domain (DID). Another essential element of the C-terminal region is the diaphanous autoregulatory domain (DAD), which can connect to the N-terminal region. The interconnection between DAD and DID region leads to the inhibition of formin function. Binding of Rho-GTPase unseal the DAD - DID interaction and the formin protein is activated.

FH2 domains accelerate the nucleation process of varying degrees. The formins nucleation promoting effect is achieved by stabilizing the unstable actin intermediates in the nucleus. One the most important features of the FH2 domain is its ability to bind to the barbed end of actin filaments in a processive manner, constantly moving with the fast-growing filament barbed ends. Another essential role of the FH2 domain is the inhibition of capping proteins connection, which enables the actin filament growing, in the presence of capping proteins.

One of the most probable functions of the formins in living cells is to protect the barbed end of actin filaments against capping proteins. In the absence of inhibitory factors capping proteins connect in less than one second to newly formed actin filaments, so the length of filaments is not even longer than 500 nm. In the presence of formins actin filaments length exceed one micrometer in mammalian cells.

2 Objectives

My work was part of a broader research for the understanding of regulatory mechanisms of the actin cytoskeleton in the Biophysics Institute at the University of Pécs.

My aim was to elaborate and optimize an appropriate spin labelling method to examine the second subunit of actin. After the paramagnetic labelling of two subunits of actin we continued our researches along the following questions:

- What kind of rotational dynamics characterize the actin subunit 1 and 2 in monomer and filamentous actin?
- How do the dynamic properties of the two subunits change in function of temperature?
- How does the connection of formin FH2 domain influence the mobility of actin subunits?
- How does the presence of formin influence the actin dynamics with changing temperature?

During my further work I planned to examine the FH2 domain of mDia1 formin using EPR, so the development of a new spin labelling method of formin was required. My aim was to answer the following questions:

- What kind of rotational dynamics characterise the FH2 domain of mDia1formin in the environment of the labelled amino acid?
- How does the formin mobility change in the presence of actin?
- How does the temperature change the dynamic properties of formin FH2?
- What is the effect of actin binding to the temperature-dependent changes of formin dynamics?

The characterization of formin FH2 domain was supplemented with differential scanning calorimetric (DSC) measurements.

3 Materials and Methods

3.1 Actin preparation

Rabbit skeletal muscle actin was isolated from the aceton-dried powder from domestic white rabbit back muscles. The actin was stored at 4°C in 4 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.1 mM $CaCl_2$ (buffer A). The concentration of G-actin was determined photometrically at 290 nm with the absorption coefficient of 0.63 $mg^{-1}mlcm^{-1}$ using a Shimadzu UV-2100 spectrophotometer. F-actin was prepared by the addition of 2 mM $MgCl_2$ and 100 mM KCl to buffer A and then incubated for 2 hours at room temperature.

3.2 Preparation of formin FH2

The FH2 domain of mammalian formin mDia1 was prepared as described previously by Shimada et al. The protein fragments were expressed in *Escherichia coli* BL21 strain. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside. Further purification of mDia1-FH2 fragments was performed with size-exclusion chromatography using Sephacryl S-300. The protein concentration was determined spectrophotometrically at 280 nm with the extinction coefficient of 20580 M^{-1} cm^{-1}. The purified formin fragments were stored at -80 °C in storing buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 5 mM DTT, 5% glycerol).

3.3 Spin labeling of actin

3.3.1 Maleimid:

Actin was labeled in F-form with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide spin label (MSL) in a molar ratio of 1:1.2 for 12 hours at 2 °C. Unreacted labels were removed by pelleting the actin by ultracentifugation. The pellet was resuspended, homogenized and dialyzed in G buffer (4 mM Tris/HCl, pH 7.6, 0.2 mM ATP, 0.2 mM CaCl2).

3.3.2 F-proxyl:

G-actin was reacted with 1.2-fold molar excess of 3-(5-fluoro-2,dinitroanilino)-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidine) (SL-FDNA) for 24 hours at 2 °C. The labeled G-actin was dialyzed against G buffer and used as SL-FDNA-G-actin or polymerized in F-buffer (4 mM Tris/HCl, pH 7.6, 0.2 mM ATP, 2 mM MgCl2). The concentration of actin in the EPR experiments was 100 µM. The labeled protein concentration was determined by comparison of the double integrals of spectra with known concentration of MSL-solution.

3.4 Spin labelling of formin FH2

The mDial-FH2 sample was dialyzed in buffer T (50 mM NaCl, 50 mM Tris-HCl, pH 7.6). The FH2 domains of formin were labeled in a molar ratio of 1:2 for 24 hours at 2 °C with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide spin label (MSL). Unreacted labels were removed by dialysis in buffer T. The amount of bound labels was determined by comparison of the labeled samples with known concentration of MSL-solution by EPR.

In some cases formin FH2 was labeled with N-ethylmaleimide (NEM). The NEM was used in 1:2 molar ratio for 24 hours at 2 °C in the storing buffer. The unreacted labels were removed by dialysis in storing buffer.

3.5 Applied methods

3.5.1 EPR spectroscopy

Electron paramagnetic resonance (EPR), also known as electron spins resonance (ESR) spectroscopy is a sensitive method to study atomic or molecular systems containing unpaired electrons. The energy states of the unpaired spins are separated into two energy states when placed in a magnetic field. An electromagnetic radiation with appropriate energy (frequency) can generate transitions between the different energy states: absorption or emission after the absorption can be measured. The energy difference between the electron states is significantly affected by the interactions of the paramagnetic centre with its molecular environment. The nuclear spins of atoms containing unpaired electron influence the energy states of electrons in the magnetic field.

This interaction between nuclear and electron spins is described as the so-called hyperfine coupling constant. The polarity of the environment changes the symmetry of electron distribution, causing a change in the hyperfine coupling constant as well.

Since most biological (macro)molecules have no intrinsic paramagnetic centres, it is necessary to attach extrinsic paramagnetic probes (spin labels) to the test molecule, even when proteins are studied. Spin labels can be attached to a particular amino acid of proteins, thus detailed information can be obtained on the specific structure of the protein subunit surrounding the labelled amino acid. The molecular conformation changes can be traced by analysing the shape of the spectrum and the position of the characteristic peaks.

The spin labelling technique commonly uses paramagnetic probes containing nitroxide free radicals (pyrrole, pyrrolidine, piperidine, oxazoline), which covalently bind to the test molecule. During my work maleimide (MSL) and F-proxyl (FDNA) paramagnetic probes were used for specific labelling of actin and formin proteins.

3.5.2 EPR measurements

Conventional and saturation transfer EPR spectra were taken with an ESP 300E (Bruker Biospin, Germany) spectrometer. First harmonic in-phase absorption spectra were obtained by using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1 or 0.2 mT. Second harmonic, 90° out-of-phase absorption spectra were recorded with 63 mW and 50 kHz field modulation of 0.5 mT amplitude detecting the signals at 100 kHz out-of-phase. The 63 mW microwave power corresponds in average microwave field amplitude of 0.025 mT in the centre region of the cell, and the values were obtained by using the standard protocol of Fajer and Marsh and Squier and Thomas. Spectra were normalized to the same number of unpaired electrons by calculating the double integral of the derived spectra. The concentration of bound labels was estimated by comparing the spectrum with EPR spectra of maleimide spin label solution of known concentration. A computer algorithm developed in our laboratory was used to obtain the double hyperfine splitting constants of spectra.

The protein samples were placed in two capillary tubes (Mettler ME-18552 melting point tubes), each of them contained 15 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 spectra were usually recorded at 23 ± 0.1 °C. Studying the EPR spectra of F-actin and F-actin samples in complex with formin as a function of temperature, the temperature was varied between 0 °C and 60 °C with an accuracy of 0.1 °C.

3.5.3 Analysis of the slow-motional EPR spectra by computer simulation

To simulate the EPR spectra in the slow-motional region, the nonlinear least-squares (NLSL) program from the Freed-groups has been used. A graphical user interface for the Windows 32-bit operating system and some modifications of the source codes necessary for the Intel FORTRAN 9.1 compiler has been developed to the 1.51b version of the NLSL software, and used earlier successfully also to simulate partially oriented slow-motional spectra for lipid systems. Among others, the NLSL software allows simulation of EPR spectra: i.) for Brownian rotational diffusion with anisotropic diffusion tensor; ii.) taking into account tilt angle between the g-factor and the rotational diffusion tensors; iii.) with maximally three different components due to molecular environments or motion. Approximate values for the hyperfine splitting constants and g-factors of the spin label SL-FDNA has been first determined by measuring the isotropic hyperfine splitting, a_o found to be 1.589 mT, and calibrating the EPR spectrum measured at 60 °C (g_o = 2.00553) against DPPH. In a series of preliminary simulations for the whole temperature interval best fits of EPR spectra resulted in the hyperfine- and g-tensors used further on in simulations: component 1.: A_{xx}, A_{yy}, A_{zz} as 0.59, 0.60 and 3.48 mT; component 2.: A_{xx}, A_{yy}, A_{zz} as 0.63, 0.63 and 3.43 mT; g-tensor: 2.00820, 2.00608 and 2.00230 for both the slow-motional components and the mobile one. Tilt angles between the g- and diffusion tensors found to be different for the two slow-motional components and depend on the temperature.

3.5.4 DSC measurements

Differential scanning calorimetry (DSC) is a sensitive method to study the thermodynamic state of proteins, protein complexes, that allows describing their spatial stability and conformational changes.

Thermal unfolding of formin, formin - F-actin complex and F-actin was monitored by a 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. Conventional Hastelloy batch vessels

were used during the denaturation experiments with 800 µl sample volume in average. Formin or F-actin buffer solutions were used as a reference sample. The sample and reference vessels were equilibrated with a precision of ± 0.1mg. There was no need to do any correction between sample and reference vessels. 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 ($C_{p,ex}$), transition temperature (T_m) and calorimetric enthalpy change (ΔH)).

3.5.4.1 Computational methods

For deconvolution of DSC traces the PeakFit 4.0 software from Jandel Scientific was used. In order to find the breakpoint of two straight lines the statistical method suggested by Jones and Molitoris was used. The method allows calculating whether the fit with two straight lines is better than a single line, and it gives the mean square error of the fit.

4 Results

4.1 EPR measurements

4.1.1 The analysis of actin labelled on Cys-374

In the first set of experiments maleimide (MSL) paramagnetic marker was used, which selectively bounds to the Cys-374 residue of actin.

Conventional EPR spectra measured on actin filaments at room temperature showed a double hyperfine coupling constant ($2A'_{zz}$) of 6.803 ± 0.010 mT (average of n = 22 measurements). The rotational correlation time calculated from this value was $\tau \sim 50$ ns, which reflect the movement of the marked actin domain.

In case of F-actin the L '/ L parameter of the saturation transfer EPR spectra was 0.91 ± 0.05 (n = 12), that corresponds to about 100 – 120 μs rotational correlation time. C '/ C diagnostic parameter of the ST-EPR measurements was 0.09 ± 0.12 (n = 12). The ST-spectral parameters of actin filaments characterize the torsion movement of several adjacent subunits.

In my further work the effect of mDia1 FH2 formin on MSL-actin was investigated. The conventional EPR measurements were performed on samples of 1: 50, 1: 25, 1: 10 and 1: 5 formin to actin molar ratio. Addition of formin to MSL-actin resulted in a decrease in the coupling constant ($2A'_{zz}$). Analysis of spectra by the line width of the low-field line as suggested by Mason and Freed showed that after binding of formin to F-actin the rotational correlation time of the label in the environment of the Cys-374 sites decreased from about 50 ns to about 30 ns at 25:1 molar ratio of actin to formin. Similarly, decreased rotational correlation times were calculated at other actin to formin molar ratios.

After adding formin FH2 to actin filaments, the amplitude growth of spectral lines characteristic to rapid rotation were observed on the conventional EPR spectra. The spectral components are made up of two parts: 1) a spectrum of bound labels reflecting the motion of the whole actin filaments; 2) the spectral contributions of loose segments. The contribution of the second component can be determined based on the difference spectra. The double integrate of the newly released spectrum component is about 10 % of the full EPR absorption.

Both diagnostic EPR parameters, L''/L and C'/C, reflected increased immobilization of labels after addition of formin to MSL-F-actin filaments. However, only moderate changes were derived in the ST EPR parameters at increasing molar ratio of formin to actin. Based on the low field L "/ L parameters the rotational correlation time of actin filament increased from 100 – 120 μs to 150 μs after formin binding. However, the rotational correlation value calculated from the C '/ C parameters increased from 7 μs to 40 μs.

4.1.2 Effects of temperature on MSL-actin and its complexes with formin

EPR technique enables to study the spin labeled proteins at different temperatures as well. MSL-labeled actin and its complex with formin FH2 were tested in the range of 0 to 60 °C.

The dependence of the calculated $2A'_{zz}$ on the 1000/T was linear in all cases, but the regression coefficient (b) is slightly different at different formin to actin ratios. At 1 : 25 formin to actin molar ratio the regression coefficients were: b = 0.392 ± 0.008 and b = 0.519 ± 0.014 for F-actin and F-actin-formin; whereas at 1 : 5 formin to actin ratio b = 0.402 ± 0.018 and b = 0.540 ± 0.022 were calculated for F-actin and F-actin-formin, respectively.

The hyperfine coupling constant of actin-formin complex was less than that of F-actin in the whole temperature range. The rotational correlation time of the covalently bound spin label can be calculated using the Goldman equation:

$$\tau = a\,(1 - 2A'_{zz} / 2A^{r}_{zz})^{b}$$

where a = 5,4 $x10^{-10}$ s és b = -1,36. The Arrhenius-relationship between the diffusion coefficient and activation energy can be applied for the temperature dependence of the rotational diffusion of molecules. Using the Arrhenius-relationship, the activation energy of the rotational diffusion can be determined. The activation energies were E = 18 kJ/mol for F-actin and E = 15 kJ/mol for F-actin-formin complex. $2A^{r}_{zz}$ = 7,055 mT was used as rigid limit. This rigid limit value was determined on F-actin samples in 40% sugar solution at - 18 °C temperature.

4.1.3 EPR measurements of FDNA-labeled actin

In order to study the 9 kDa sudomain 2 of actin, FDNA paramagnetic label was used, which can selectively bind to the Lys-61 residue.

The outer splitting of the attached SL-FDNA label on F-actin was significantly smaller ($2A'_{zz}$ = 6.121 ± 0.021 mT, n = 21) than the splitting of MSL-F-actin.

On the conventional EPR spectrum of FDNA-labeled F-actin a broad high field component can be observed, that suggests that the spin label attached to the Lys-61 residue has either two conformations possessing different rotational dynamics, or one fraction of the labels locate on a different site, for example on Lys-113 residue. In contrast, in the G-form of SL-FDNA-actin only one hyperfine splitting value could be derived, so the probe is very likely attached to only one location in the monomer. In this case the mean value of the $2A'_{zz}$ was 5.912 ± 0.05 mT (n = 7).

Using the averaged rigid limit of the hyperfine splitting constant obtained from spectral simulation (6.915 mT), the apparent rotational correlation time for the motion of the SL-FDNA label on G-actin was estimated to be about 8 – 9 ns. This value is shorter than the rotational correlation time of the whole monomer molecule (~ 18 ns), indicating that the probe reflects the subdomain motion of the actin monomers.

After the addition of formin to SL-FDNA labeled F-actin the conventional EPR spectra showed increased mobility in the nanosecond time range, according to the smaller coupling constant and a new spectral component. This mobile fraction was almost independent of the formin to actin molar ratio below 0.1, and the double integral of this fraction was always smaller than 5 % of the total actin concentration. Binding of formin to actin induced conformational changes, which resulted in the increased mobility of the environment of Lys-61 amino acid. In contrast, ST-EPR measurements on SL-FDNA-F-actin and F-actin-formin at actin concentrations of 120 – 150 μM showed an increase of the diagnostic parameters L''/L and C'/C, indicating a decreased mobility of actin filaments.

4.1.4 Temperature-dependent EPR measurements on FDNA-actin

The conventional EPR spectra of FDNA labeled actin and actin-formin samples resulted in a sigmoid relationship between the coupling constant and the reciprocal absolute

temperature. However no differences were found between the temperature dependence of formin-actin and actin samples. The conventional spectra measured at 0 and 60 °C showed two additional spectral components in both cases, but only one of them determined the measured hyperfine coupling constant. This assumption is also supported by the results of spectral simulations presented later. A sigmoid Boltzmann function was attempted to fit the temperature dependence of the external peaks:

$$2A'_{zz}(z) = [(2A'_{zz,\,min} - 2A'_{zz,\,max}) / (1 + \exp(z - z_0 / \delta z))] + 2A'_{zz,\,max}$$

where z_0 is the inflection point and δz is the growth rate. $2A'_{zz,min}$ and $2A'_{zz,max}$ are the minimum and maximum hyperfine coupling constants obtained during the measurements. The best fit for z = 1000 / T; z_0 = 3.256 (34 ° C); δz = 0.155 parameters were found at $2A'_{zz,min}$ = 5.10 mT and $2A'_{zz,max}$ = 6.94 mT values. Simulation of EPR spectra measured at different temperatures showed that none of the spectra can be described by a single slow-motional component, the mobile component was shown in each experimental spectrum, but it became negligible below 30 °C. In addition to the spectrum component in the fast rotational range, two different components with slower correlation times had to be taken into account to simulate the experimental spectra in all cases; a single isotropic Brownian rotational diffusion could not describe the rotation of the Lys-61-labeled F-actin.

4.1.5 EPR measurements on mDia1 FH2 formin

Unlike the G- and F-actin the EPR spectra of maleimide labeled formin FH2 showed inhomogeneous distribution of the markers; both weakly and strongly immobilized molecules were detected by EPR. The two elements of the population in the experimental spectra could be separated by computer analysis. Approximately 60% of the spin probes were in strongly immobilized state. The hyperfine coupling constant of MSL-formin was $2A'_{zz}$ = 6.538 ± 0.044 mT (n = 4) at room temperature. The strongly immobilized label on formin had a rotation correlation time of 25 ns, while for the weakly immobilized label 3.5 ns was calculated. Addition of F-actin to MSL-formin resulted in the increase of coupling from 6.538 to 6.727 mT as the actin to formin molar ratio raised from zero to 1 : 1, and 6.640 ± 0.032 mT (n = 7) at 5 : 1 formin-actin molar ratio. The effect of formin binding to actin seemed to be independent of the proteins molar ratio regarding the labeled formin segment.

4.1.6 Temperature-dependent EPR measurements on MSL-formin

The conformation of spin labeled formin FH2 was examined by EPR measurements at different temperatures in the range between 0 and 60 °C in 5 °C increments. Rising temperature increased the population ratio of the mobile EPR component (indicated by the relative amplitude of the peak I_{+1}), while the hyperfine coupling constant of the immobilised population declined. Plotting the coupling constant in function of reciprocal temperature, a breakpoint was observed at around 40 °C, suggesting a conformational change in the protein. The rotational correlation time was determined by the method of Goldman, Bruno and Freed using $2A^{r}_{zz}$ = 7.104 mT rigid limit value. According to the results of the temperature dependent measurements a breakpoint occurred at around 42.8 °C. The activation energy before and after the breakpoint was different; the calculated activation energies were 23.5 kJ / mol and 14.6 kJ / mol. These results do not rule out the possibility that a conformational change occurs with the formin-actin interaction, which covers only smaller segments of formin. To further analyze the EPR data we calculated the ratio of I_{+1}/I_m, where I_m is the peak height of the low-field maximum in the spectrum of the MSL-formin, and I_{+1} is the peak-to-peak height of the first component of the spectrum characterizing the weakly immobilized labels. The function of I_{+1}/I_m against reciprocal absolute temperature can be approximated with a near exponential function without any break. In the presence of actin the decay constant of the exponential function was significantly smaller showing that the binding of formin to actin affected the ratio of the two populations characterized with different rotational mobility. The value of the decay constant depended on the ratio of formin to actin, evidencing that the two populations of the bound labels are mostly determined by the contact of formin dimers to F-actin.

Using computer manipulation we could calculate the ratio of the two components double integrals, i.e. the relative contributions of the components in the m = +1 EPR transition, depending on temperature. A_{im} represents the double integral of the spectral component characterizing the strongly immobilized labels at the low-field maximum, whereas A_m is the double integral of the first component of the spectrum characterizing the weakly immobilized labels. The A_{im}/A_m ratio - which determines the equilibrium constant (K) at given temperature - shows an exponential dependence as a function of reciprocal absolute temperature. The free enthalpy change (ΔG) was 6.7 kJ / mol at T_m = 20 °C. Using the relation $\Delta G = \Delta H - T\Delta S$, the entropy (ΔS) and enthalpy changes (ΔH) can be obtained.

According to the Van't Hoff plot, the change in Gibbs free energy can be considered constant in this temperature range, which characterizes the immobile ↔ mobile transformation in the interaction of actin and formin.

4.1.7 DSC measurements

The DSC transition of Formin (FH2) and formin-actin complexes was calorimetric irreversible. The melting temperature (T_m) of formin was 43.1 °C. The calorimetric enthalpy was ΔH = 104 kJ / mol, the entropy change ΔS = 0.33 kJ / molK at T_m temperature. The Gibbs free energy change was ΔG = 7.7 kJ / mol at t = 20 ° C. Using the PeakFit 4.0 program (Jandel Scientific) the excess heat capacity ($C_{p,ex}$) of the thermal transition was well approximated with an exponentially modified Gaussian curve (EMG function). The maximum of the function was at T_m = 43.19 °C, the width at half maximum occurred to be dW = 4.95 °C.

DSC thermograms acquired during the measurements were compared to simulated curves according to the method of Conjero-Lara et al. The parameters of simulated temperature curves - melting temperature, width at half maximum - approximated the measured transitions well, taking into account the signal / noise ratio of the experiments. The transition temperature of F-actin was T_m = 68.2 ° C. Addition of formin FH2 to F-actin reduced the transition temperature: T_m of the protein complex was 1.5-2 ° C lower than that of formin-free actin samples. These results were in agreement with previous observations, that the binding of formin to actin filaments decreases the thermodynamic stability of actin.

By changing the molar ratio of formin to F-actin, the transition temperature of the complex changed. As the actin to formin molar ratio was 3 : 1, the T_m = 66.5 ° C; at 5 : 1 ratio T_m = 65.1 ° C; at 10 : 1 ratio the value was close to the transition temperature of formin free F-actin, T_m = 68.2 ° C. The shape of the DSC curve of formin-actin complex indicates that the transition is not a simple two-state process, more likely it includes the sequential or concerted unfolding of several interacting domains.

The asymmetry of the transition curves may arise from the summation of quasi-independent units, resulting uncoupling between the structural domains. This may be due to the temperature-induced conformational change occurring in the protein consisting of several domains, or the altered interaction between the subunits of the protein complex.

5 Conclusions

Conventional and saturation transfer EPR was used to follow the local and global conformational changes in actin and formin occurring during their interactions. Paramagnetic labelling of the actin Cys-374 and Lys-61 residue enabled to study and compare the conformation of the first and second actin subunit and to examine the changes induced by the binding of formin FH2 domain.

The conventional spectrum of FDNA-actin showed a smaller coupling constant than the spectrum of MSL-actin, indicating a more relaxed conformation of the second subunit. The addition of formin to F-actin resulted in a decrease of the coupling constant by both labeled actin samples. For MSL-actin a more pronounced change was observed that may arise from a more rigid structure of the environment of the MSL-labels. The coupling constant for both label changed only slightly with different formin to actin ratio.

The mDia1 FH2 binds to the side of actin filaments as well, but with weak binding affinity. Barbed end of actin has much higher affinity for formin (20-50 nM). Taking into account the low formin-actin concentration ratio, we can exclude that the formin binding the filaments sides caused the observed effects. Our results are in agreement with the fluorescence measurements as previously reported, which showed an increase of actin protomers flexibility due to formin binding.

According to the temperature-dependent EPR measurements with MSL-actin and F-actin-formin complex, the hyperfine coupling constant showed a linear correlation with reciprocal absolute temperature. In case of actin-formin complex the hyperfine coupling constant was less than the measured for F-actin in the entire range from 0 to 60 ° C and the activation energy of the complex was less than that of MSL-actin. These results support the previously reported DSC results, that formin binding reduces the denaturation temperature of actin filaments.

Paramagnetic labeling of the FH2 domain of formin allowed studying the effect of formin-actin interaction on formin. From the conventional EPR spectra of MSL-formin two hyperfine coupling constant values could be determined with rotational correlation times of 25 ns, and the smaller fraction with 3.5 ns at room temperature. The longer correlation time may characterize the movement of the formin FH2 monomer. Formin dimers are able to move together with the positive end of growing actin filaments in a

processive manner. Accordingly, the flexible connection between the formin monomers can be a key to formin function.

The temperature-dependent EPR measurements showed, that of the hyperfine coupling constant decreased, the ratio of mobile component increased with increasing temperature. The temperature dependence of the coupling constant suggests a conformational change around 41 °C. Formin structure loosening was also supposed by the activation energy decrease above the transition temperature. Addition of F-actin to MSL-formin resulted in the growth of the coupling constant in the entire temperature range, which indicates the formin structure to become more rigid. The temperature dependence of the coupling constant of MSL-formin-actin complex showed a break point at 44.2 ° C.

Thermodynamic characterization of formin mDia1 FH2 was supplemented with differential calorimetric assays. The transition temperature of formin FH2 was 43.1 °C. The transition temperature of actin-formin complex was close to the characteristic value of F-actin, which showed good agreement with previously reported DSC results. Typical denaturation transition of formin was not detected in the case of actin-formin samples. This could be due to the low concentration of the formin samples, or the stabilizing effect of actin on formin can cause a shift of the transition temperature above 60 ° C.

The differences between the temperature-dependent EPR and DSC results of the difference in the sensitivity of the two methods: the conventional EPR reflects segmental movement in the environment of the spin labeled amino acid, while the DSC transitions are characterized by thermodynamic changes of the entire protein or protein complex. By using both methods the local and the global structural and dynamic properties of formin and formin-actin complex can be investigated.

The thesis is based on the following publications

Tünde Kupi, Pál Gróf, Miklós Nyitrai and József Belágyi: The uncoupling of the effects of formins on the local and global dynamics of actin filaments. Biophys J. Vol. 96 (2009), 2901-2911

Tünde Kupi, Pál Gróf, Miklós Nyitrai and József Belágyi: Interaction of formin FH2 with skeletal muscle actin. EPR and DSC studies. Eur Biophys J. Vol. 42 (2013), 757-765

Other publications

Réka Dudás, **Tünde Kupi,** Andrea Vig, József Orbán and Dénes Lőrinczy: Effect of Phalloidin on the Skeletal Muscle ADP-actin Filaments. Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 3, 709 – 712.

Andrea Vig, Réka Dudás, **Tünde Kupi,** József Orbán, Gábor Hild, Dénes Lőrinczy and Miklós Nyitrai: Effect of Phalloidin on Filaments Polymerized from Heart Muscle ADP-actin Monomers. Journal of Thermal Analysis and Calorimetry, Vol. 95 (2009) 3, 721 – 725.

Tamás Deák, **Tünde Kupi,** Róbert Oláh, Lóránt Lakatos, Lajos Kemény, György D. Bisztray, Ernő Szegedi: Candidate plant gene homologues in grapevine involved in Agrobacterium transformation. Cent. Eur. J. Biol. 8(10), (2013), 1001-1009

Tünde Kupi, Tamás Deák, Ernő Szegedi: Detection of self-complemetary inverted repeats by a single forward primer driven PCR. Acta Biologica Szegediensis, Vol. 58. (2014)