Ph.D. THESIS

INVESTIGATIONS OF THE MECHANISMS UNDERLYING SARCOMERIC FILAMENT ASSEMBLY

Tamás Huber

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Medical School
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Tamás Huber

Program:	Interdisciplinary Medical Sciences Doctoral School
Leader of the Doctoral School:	Dr. Balázs Sümegi
Subprogram (B-130):	Investigating functional protein dynamics using biophysical methods
Program leader:	Dr. Miklós Nyitrai
Supervisors:	Dr. Miklós S. Z. Kellermayer and Dr. László Grama

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INTRODUCTION

The structural and functional unit of the striated muscle is the sarcomere. Although a lot is known about the molecular mechanisms of the active contraction of the sarcomere, it is still not exactly clear how this impressively ordered structural complex is formed and which processes determine and regulate the conformation and interaction of its filaments. Based on present-day knowledge both proteins, titin and formin, examined in this work play an important role in the regulation of the sarcomere structure.

Titin is the largest protein in nature. One of its major functions is to maintain the passive elasticity of the muscle, a feature that is independent of the thin and thick myofilaments. Titin's i*n situ* average length is more than 1 μm with a molecular weight of 3-3.7 MDa, depending on the isoform [1, 2]. The giant titin molecule is the third most abundant protein of the vertebrate striated muscle, after myosin and actin. In the striated mucle single titin molecules span half of the sarcomere parallel with thin and thick filaments [3]. The N-terminal part of the molecule is anchored to the Z-disk, the C-terminal is tightly bound to the M-line. According to the sequence analysis it is extensively modular in structure. Approximately 90% of its mass consists of repeating immunoglobulin (Ig) and fibronectin III (FNIII) domains [4]. The remaining part is organized in 17 unique, nonrepetitive sequence motifs which are situated between the Ig and FNIII repeats.

One of the most studied and functionally significant unique sequences is the PEVK domain, enriched in proline (P), glutamic acid (E), valine (V), and lysine (K) residues. Depending on the isoform its length ranges from 163 to 2174 residues [5]. Two distinct types of repeat motifs have been observed in the skeletal muscle PEVK segment: PPAK repeats are 26–28-residue motifs that often begin with the amino acids PPAK and polyE motifs display a preponderance of negatively charged glutamate residues.

Although the elasticity of the PEVK region of titin has been extensively studied, its background is not yet fully understood. It has been proposed that in case of low to moderate stretch forces, the force-extension relationship could be well fitted with a wormlike chain (WLC) model of entropic elasticity. In case of higher stretch forces enthalpic contributions are also likely to appear [6, 7]. Such enthalpic contributions have been proposed to originate from electrostatic and perhaps hydrophobic interactions within the PEVK segment.

The sarcomeric thin filaments are assembled upon actin polymerisation. Formins play a critical role in this process. Formin proteins are similarly to the titin multi-domain molecules. Molecular-level, *in vitro* studies revealed that formins belong to the family of actin-regulating nucleation factors [8-10]. Interestingly, in addition to their role in actin nucleus stabilization they also regulate actin elongation in many ways [11, 12].

In our work we investigated a member of the DAAM (Dishevelled Associated Activator of Morphogenesis) protein family, that is a recently discovered and less characterized formin family [13]. A characteristic property of its members is that the two domains located at the ends of the molecule regulate the activity of the protein. Based on current knowledge their *in vivo* regulator is the Rho GTPase protein family. In the absence of an interacting partner the DID (Diaphanous Inhibitory Domain) and DAD (Diaphanous Autoregulatory Domain) domains can bind to each other and this interaction keeps formin in the inactive form. When GTPase binds to the N-terminally located GTPase binding domain the molecule loses the autoinhibited conformation and the domains which are responsible for the interaction with actin become accessible [14]. One of these interacting domain is the FH1 (Formin Homology 1) domain, which does not bind directly to actin but interacts with profilin, an actin monomer binding protein. The FH2 (Formin Homology 2) domain is the most conserved part of the molecule and it is the primary actin binding site of the formin [15].

In the last years it turned out that the C-terminally located DAD domain of the molecule does not only play a role in the autoinhibition. In 2011 Gould *et al.* showed that this part of the molecule has a dual role: it possibly binds to actin and also enhances the nucleation behaviour of the formin [16].

Based on these observations our aim was to examine the C-terminal part of the DAAM formin and study the difference between the already well-known FH2-actin and FH2-DAD-actin interactions.

OBJECTIVES

Earlier studies described titin's PEVK region as an unstructured polypeptide chain. However, other observations indicated that secondary structural features such as polyproline-type II helices may be present within the PEVK domain. In this work, we examined the random coil nature of *m. soleus* titin PEVK by testing the predictions of statistical polymer chain models.

Our particular aims were:

- to determine the end-to-end distances of fluorescently labeled, synthetic PEVK peptides by using fluorescence resonance energy transfer measurements;
- to describe the bending rigidity and conformation of the peptides through defining their apparent persistence lengths;
- to examine the effect of chemical denaturants on the conformation;
- to examine the effect of ionic strength on the conformation of peptides;
- to investigate the chain flexibility through temperature-induced fluctuation effects;
- to explore whether secondary structural features may be present within the peptides by using molecular dynamic simulations;
- cloning, expression, purification and nanomechanical characterization of recombinant PPAK fragment with single-molecule atomic force microscopy.

In the other part of my work we attempted to characterize the C-terminal region of *Drosophila* DAAM. Our specific aims were:

The preparation of the following DAAM formin constructs:

- GST-FH1-FH2-DAD+C-terminal *(later on: CDAAM)*
- GST-DAD *(later on: DAD)*
- GST-DAD+C-terminal *(later on: DAD+C)*
- wild-type and mutant GST-FH1-FH2 *(later on: FH1-FH2)*

To examine, whether

- CDAAM is able to increase further the actin assembly rate compared to FH1-FH2,
- which phase of the polimerisation is affected,
- DAD/DAD+C constructs influence the kinetics of actin polimerisation,
- DAD/DAD+C domains of DAAM formin are able to bind actin monomers,
- the C-terminally located amino acids of the molecule play a role in the binding,
- the I732A mutation abolishes the nucleation activity of the FH2 domain (as previously reported *in vivo*).

MATERIALS AND METHODS

Preparation of peptides and proteins

PEVK peptides

Eleven- and twenty-one-residue-long PEVK peptides were produced by our collaborator, Dr. Lívia Fülöp and colleagues with solid-state synthesis as described previously [17]. The sequences of the peptides correspond to regions 17413–17442 (PEVK11) and 17413–17472 (PEVK21) (GenBank accession no. X90569 (version X90569.1)) within the titin sequence, respectively [5].

Preparation of human skeletal PPAK fragments

Human skeletel muscle cDNA library was a generous gift of Dr. Siegfried Labeit. The PPAK fragment in this work was expressed from the N-terminal third of *m. soleus* titin PEVK domain. The 603-nucleotide-long fragment (17413-18015, GenBank accession no. X90569 [5]) was cloned into pET-28a vector (Novagen) between NheI and XhoI restriction sites (Promega) introduced independently with PCR by using specific oligonucleotides. The construct was transformed into *E. coli* strain BL21(DE3)pLysS (Promega) and expressed in Luria-Bertani broth (Scharlau) for 3 hours after induction with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside, Sigma).

The cell lysate containing the expressed protein was centrifuged (Sorvall Ultra Pro 80 ultracentrifuge, T-1250 rotor, 100000 g, 4 °C, 1h). His_6-tagged proteins were purified on Ni-NTA Agarose column under native conditions following manufacturer's instructions (Qiagen). Finally, the eluted protein were dialyzed against phosphate buffer and stored at -80 °C until later use.

Expression and purification of DAAM constructs

The pGEX-2T cloning vectors containing the DAAM formin sequences of *Drosophila melanogaster* were generous gift from Dr. József Mihály and his workgroup (Biological Research Centre, Institute of Genetics, Szeged). Different DAAM constructs were prepared based on previously described methods [18].

Preparation and fluorescent labelling of actin

Acetone-dried powder of rabbit (*Oryctolagus cuniculus*) skeletal muscle was obtained using a preparation technique described previously [19]. Actin was prepared from the muscle powder according to the method of Spudich and Watt [20].

In order to follow the polymerisation, cysteine-374 of actin was labelled with N-(1-Pyrene)-iodoacetamide (pyrene, Molecular Probes) [21].

In case of steady-state fluorescence anisotropy and total internal reflection fluorescence microscopy (TIRFM) experiments actin was conjugated with Alexa Fluor 488-succinimidyl-ester (Alexa 488, Molecular Probes) based on previously described methods [22].

Fluorescence spectroscopy measurements

Measurement of actin polimerisation

The effect of DAAM constructs on the polimerisation was followed by using pyrene-labelled actin. The experiments were carried out in the presence of 2.5 μM actin (5% pyrene labelled) at 20 °C with a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer. The excitation and emission wavelengths were 365 nm and 407 nm, respectively. Excitation and emission slits were both set to 5 nm. The elongation rate was determined from the slope of the linear fit to the pyrene fluorescence curves at half-maximum polymerisation.

Steady-state fluorescence spectroscopy measurements on PEVK peptides

Fluorescence spectra of PEVK peptides were measured using a Fluorolog-3 spectrofluorometer. In these experiments we measured the fluorescence resonance energy transfer (FRET) between the N-terminally located tryptophan (donor) and the C-terminally located IAEDANS (acceptor). Tryptophan was excited at 295 nm and emission was recorded between 305 and 700 nm. Excitation and emission slits were both set to 5 nm.

The FRET efficiency (E) was calculated as:

$$E = 1 - \frac{F_{DA}}{F_D}, \tag{1}$$

where F_{DA} and F_D are the corrected donor fluorescence intensities in the presence and absence of the acceptor, respectively.

The distance between the donor and acceptor fluorophore (R) was calculated as:

$$R = \sqrt[6]{\frac{R_0^6}{E} - R_0^6}\,, \tag{2}$$

where R_0 is Förster's critical distance at which transfer efficiency is 50%. The R_0 value of the tryptophan-IAEDANS pair is 2.2 nm, as reported previously [23].

In the case of temperature-dependent measurements, the f' parameter (FRET efficiency normalized with the donor emission intensity) was calculated as:

$$f' = \frac{E}{F_{DA}}\,. \tag{3}$$

The temperature dependence of f' characterizes the average flexibility of the protein matrix between the donor and acceptor [24].

Fluorescence lifetime experiments on PEVK peptides

In case of some peptide samples FRET measurements were carried out with time-correlated single photon counting (TCSPC) technique as well. These experiments were performed by using a ISS Chronos-BH spectrometer at 22 °C. Average fluorescence lifetimes (τ) were calculated from the results of the analysis assuming discrete lifetime distribution:

$$\bar{\tau} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}\,, \tag{4}$$

where α_i and τ_i are the i^{th} preexponential factor and lifetime component of tryptophan displaying a complex decay [25].

FRET efficiency was calculated from the determined fluorescence lifetimes as:

$$E = 1 - \frac{\overline{\tau_{DA}}}{\overline{\tau_D}}\,, \tag{5}$$

where τ_D and τ_{DA} are the donor (tryptophan) lifetimes in the presence and absence of the acceptor (IAEDANS), respectively.

Steady-state fluorescence anisotropy measurements on DAAM formin fragments

The measurements were carried out with Horiba Jobin Yvon Fluorolog-3 spectrofluorometer at 20 °C. The experiments were carried out in the presence of 0.5 μM actin (50% Alexa Fluor 488 labelled). The concentration of the DAD/DAD+C contructs was depending on the given experiment. The fluorescently labelled actin monomers containing samples were irradiated with plane-polarized light at 488 nm wavelength, and the degree of polarization of the emitted fluorescence was analysed at 516 nm. Excitation and emission slits were both set to 5 nm. The steady-state fluorescence anisotropy can be calculated from the measured intensities (I_{VV} and I_{VH}) as [26]:

$$\mathrm{r}=\frac{I_{VV}-GI_{VH}}{I_{VV}+2GI_{VH}}, \tag{6}$$

where the first and the second subscripts refer the orientation of the excitation and emission polarizers, respectively (*V*: vertically, *H*: horizontally); and *G* is a correction factor which is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light.

The steady-state fluorescence anisotropy values (*r*) were plotted as a function of the concentration of the investigated proteins. The equilibrium dissociation constant (*K*) was calculated as:

$$\frac{r-r_A}{r_{AD}-r_A}=\frac{A_0+D_0+K-\sqrt{A_0+D_0+K^{\,2}-4A_0D_0}}{2}, \tag{7}$$

where r_A and r_{AD} are the anisotropy values in the absence and in case of saturation concentration of the examined proteins, respectively; A_0 and D_0 are the total concentration of actin and DAAM constructs, respectively.

Microscopy experiments

Total internal reflection fluorescence microscopy

The nucleation and elongation phase of actin polymerisation can not be distinguished by using pyrene-actin-based fluorescence spectroscopy measurements. To elucidate this problem, we also performed total internal reflection fluorescence microscopy (TIRFM) experiments.

The measurements were carried out with an Olympus IX81 epifluorescence microscope equipped with a TIRF unit at room temperature.

The elongation rate (v) measured in μm/s was calculated as:

$$v = \frac{\Delta l}{\Delta t} = \tan \alpha \,, \tag{8}$$

where *tan α* is the slope of the filament length (Δl) – time (Δt) function. Assuming that a 1-μm-long filament contains 330 subunits, the association rate (k_+) of actin monomers can be calculated as follows:

$$k_+ = \frac{v}{F\ G - cc} \,, \tag{9}$$

where in case of one filament F = 1, G is the total G-actin and cc the critical concentration (cc=0.15 μM, as reported previously [27]).

Atomic force microscopy

The PPAK fragments were mechanically stretched by using an atomic force microscope (AFM) dedicated for molecular manipulation (MFP1D; Asylum Research, Santa Barbara, CA, USA).

The force versus displacement curves were plotted with the wormlike chain (WLC) model of entropic elasticity [28, 29]:

$$\frac{FL_p}{k_B T} = \frac{R}{L_c} + \frac{1}{4\ 1 - R / L_c\ ^2} - \frac{1}{4} \,, \tag{10}$$

where L_p a is the effective persistence length, k_B is the Boltzmann constant, L_c is the effective contour length, R is the end-to-end distance, T is the absolute temperature and F is the external force.

Polymer model calculations

The contour lengths (L_c) of the PEVK peptides were calculated as:

$$L_c = N_{aa} \cdot L_{aa} + L_{IAEDANS} \ , \tag{11}$$

where N_{aa} is the number of residues in the peptide, L_{aa} is the residue spacing (0.38 nm) and $L_{IAEDANS}$ is the size of the acceptor molecule (0.87 nm, [30]). Contour lengths obtained this way were 4.18 nm and 7.98 nm for PEVK11 and PEVK21, respectively.

The contour length of the PPAK fragment was calculated based on the amino acid sequence, which is 85.12 nm (224 × 0.38 nm).

The average end-to-end length (R) of the peptides was determined from FRET efficiencies as described above (**equation 2**). Apparent persistence length (L_p), measure of the bending rigidity of the polymer chains, was obtained numerically by using the formula for the mean-square end-to-end distance R^2 , assuming that the conformation of peptides is described by the WLC model [31, 32]:

$$\left\langle R^2 \right\rangle = 2L_p L_c \left(1 - \frac{L_p}{L_c} \left(1 - e^{-\frac{L_c}{L_p}} \right) \right). \tag{12}$$

Molecular dynamics simulations

The molecular dynamics simulations were performed by our collaborator, Dr. Csaba Hetényi. The GOR4 [33], NetSurfP [34], Jpred3 [35] and PSIPRED [36] secondary structure prediction servers were applied to get an estimate for the possible secondary structures of the nonmodified peptides.

RESULTS

Spectral properties of PEVK peptides

In the case of the IAEDANS-labelled peptides the absorption spectra displayed a significant peak at 336 nm, which corresponds to the absorption maximum of the acceptor. The spectra exhibited a peak at approximately 280 nm characteristic for the absorption of proteins.

Comparison of the fluorescence emission spectra of the respective unlabelled and IAEDANS-labelled PEVK peptides confirmed the presence of energy transfer. The intensity of the tryptophan donor emission in the presence of the acceptor (F_{DA}) was much reduced compared to that in the absence of the acceptor (F_D).

Apparent persistence length of PEVK peptides

To characterise the bending rigidity of the peptides we calculated their apparent persistence lengths. The donor-acceptor distance, corresponding to the end-to-end distance of the peptides, was determined by measuring the decrease of donor fluorescence in the presence of the acceptor (**equation 2**). Under conditions corresponding most closely to the physiological (0.2 M ionic strength and at 20 °C), our experiments revealed average end-to-end distances (R) of 2.12 (±0.01) for PEVK11 and 2.69 (±0.05) nm for PEVK21. These values and the sequence based contour lengths obtained with **equation 11** were used to calculate the apparent persistence length. The calculated apparent persistence lengths (L_p) were 0.63 for PEVK11 and 0.48 nm for PEVK21 (**equation 12**).

Effect of chemical denaturants on PEVK peptides

We performed experiments to assess whether there are interactions within the PEVK peptides that might be disrupted with chemical denaturants. We studied at first the effect of guanidine hydrochloride (GuHCl) on the two peptides, but results were similar when urea was used. Upon adding the chemical denaturant, FRET efficiency decreased gradually and resulted in a decrease of transfer efficiency from 0.56 (±0.01) to 0.41 (±0.02) and from 0.23 (±0.02) to 0.16 (±0.003) in the case of PEVK21 and PEVK11, respectively. Notably, in the case of PEVK21 the FRET efficiency decreased more steeply in the range of 2–4 M, than in the rest of the denaturant concentration

range. The changes in transfer efficiency correspond to an increase of polymer-chain end-to-end distance (R).

Considering that the Förster's critical distance for the tryptophan-IAEDANS donor acceptor pair is known (R_0=2.2 nm, [23]), we calculated the end-to-end distances from the corresponding transfer efficiency values (**equation 2**). As a result, we obtained an increase of R from 2.12 (±0.01) to 2.34 (±0.03) nm for PEVK11 and from 2.69 (±0.05) to 2.91 (±0.01) nm for PEVK21. The calculated apparent persistence length values increased by ~0.18 nm for PEVK11 and ~0.09 nm for PEVK21 in the range of 0 to 6 M GuHCl concentration.

Fluorescence lifetime measurements on PEVK peptides

To validate the transfer-efficiency results obtained in steady-state fluorescence experiments and to resolve structural or dynamical details, time-domain fluorescence lifetime measurements were carried out at distinct denaturant concentrations. Double-exponential fits gave the best results, suggesting that two populations contribute to the fluorescence behavior. Upon GuHCl addition, only the longer lifetime shortened significantly.

The transfer efficiencies obtained from average lifetime measurements are comparable to those obtained in steady-state measurements (the maximum difference is within 4.2%).

Effect of ionic strength on the conformation of PEVK peptides

To investigate the effect of ionic strength on the conformation of peptides, we recorded fluorescence emission spectra at varying KCl concentrations and calculated the FRET efficiencies. Energy transfer efficiency increased by 6% in the case of PEVK11. In the case of PEVK21, a greater increase in transfer efficiency was observed (10.3%) upon increasing the ionic strength.

We also examined the effect of calcium on the transfer efficiency of the peptides. Upon increasing the calcium concentration from pCa 9 to pCa 2 (pCa: -log[Ca^{2+}]), we observed no significant change in FRET efficiency (<2% increase).

Effect of temperature on the conformational dynamics of PEVK peptides

To further investigate the conformational dynamics and fluctuations of the peptides, we carried out temperature-dependent fluorescence resonance energy transfer measurements. Increasing the temperature from 10 to 50 °C led to an increase in FRET efficiency (from 20.7 to 24.2%) for PEVK21, but only small changes for PEVK11. Considering that increasing temperatures may be used to perturb molecular structure via increase in molecular fluctuations, the structural dynamics of the PEVK peptides was characterised by measuring the normalized FRET efficiency (relative f', **equation 3**) across the temperature range of 10–50 °C. Upon increasing temperature, the relative f' increased by 30% and 70% for PEVK11 and PEVK21, respectively.

The trajectories of the f' change were different in the case of the two peptides: whereas for PEVK11 the f' function leveled out, in the case of PEVK21 a steep increase was observed followed by a departure from the original trend at 40 °C.

Molecular dynamics calculations on PEVK peptides

To investigate whether residual structural features persist in the PEVK peptides, we carried out 100-ns-long molecular dynamics simulations for each peptide. The molecular dynamics simulations were performed by our collaborator, Dr. Csaba Hetényi. For PEVK21, all servers predicted an α-helical structure for the core residues, whereas for PEVK11 either helical or random coil structure or their combinations were assigned by different secondary structure prediction servers. During the first 10 ns of the simulations the initial α-helical structure disappeared partially (PEVK21) or completely (PEVK11), and loose π-helical, bend, or turn motifs appeared.

After the energy minimization, the distances between the aromatic side chains of the N-terminal tryptophan and the C-terminal IAEDANS-modified cysteine residues were 2.2 and 2.9 nm, in the case of PEVK11 and PEVK21, respectively.

Single-molecule force spectroscopy on PPAK fragments

The elasticity of PPAK fragments was studied by using single-molecule force spectroscopy. We collected force versus displacement curves in repeated stretch and release cycles. The non-linear release curves curves were fitted with the wormlike chain equation (WLC, **equation 10**).

Based on the WLC-fit the mean effective contour and persistence length of the PPAK fragment were 81 (±13.3) and 0.68 (±0.27) nm, respectively. The contour length obtained from the AFM experiments is comparable with the sequence-based theoretical value (85 nm, **equation 11**). This result means that during the mechanical manipulation the molecules were stretched between their ends.

CDAAM further increases the polymerisation rate of actin compared to the FH1-FH2 construct

In this experiments we investigated how the presence of DAD (characterised as an autoinhibitory domain) affects the interaction between formin and actin.

Two DAAM constructs were used: the previously described FH1-FH2 domain regions [27], and the CDAAM. The kinetics of the actin polymerisation was followed by using pyrene-labelled actin.

The analysis of our data revealed that the CDAAM can further increase the polymerisation-enhancing effect of the FH1-FH2 domains. Interestingly, neither the DAD+C nor the DAD construct affected the assembly rate of actin filaments in the investigated concentration range. According to our measurements the mutated FH1-FH2 construct does not have any effect on the polymerisation of actin.

Effect of CDAAM and FH1-FH2 constructs on specific phases of actin polymerisation

A detailed investigation of how actin polymerisation is affected by DAAM constructs was carried out by total internal reflection fluorescence microscopy (TIRFM) measurements. The analysis of the microscopic images showed that in the presence of both FH1-FH2 and CDAAM the number of actin filaments notably increased compared to the control.

According to the results, both constructs are able to catalyze the nucleation of actin filaments. The elongation rate of filaments was determined from the time-dependent change of the actin filament length. We obtained a spontaneous elongation rate of 2.99 (±0.12) subunit/s for actin filaments (**equation 9**), that is in agreement with previously published data [27]. The elongation rates of actin filaments in the presence of FH1-FH2 and CDAAM were 0.30 (±0.08) and 0.87 (±0.59) subunit/s, respectively. Thus, both constructs inhibit the association of actin monomers at the barbed end, but this effect is less pronounced in case of the CDAAM construct. DAD+C, DAD and the mutated FH1-FH2 exhibited negligible effect on the elongation of filaments, in agreement with the results of pyrene-actin-based fluorescence spectroscopy experiments.

The DAD and DAD+C constructs bind to actin monomer

To assess whether DAD domain can bind to actin monomer, steady-state fluorescence anisotropy measurements were carried out by using Alexa 488-labelled actin.

We found that both constructs bind to labelled-actin monomers, but to completely different extents. The affinity of the constructs to actin monomers was determined using **equation 7**. In case of the construct lacking the C-terminus a much weaker affinity was measured ($K=43.4 \pm 1.0\ \mu M$) than in case of DAD+C ($K=0.76 \pm 0.09\ \mu M$).

SUMMARY, CONCLUSIONS

In the first part of our work, we investigated the conformation of *m. soleus* titin PEVK domain by using 11- and 21-residue-long synthetic PEVK peptides and a 224-residue-long PPAK fragment.

- We calculated the end-to-end distances (R) of the peptides by carrying out fluorescence resonance energy transfer measurements. Under conditions corresponding most closely to physiological, our experiments revealed R-values of 2.12 (±0.01) for PEVK11 and 2.69 (±0.05) nm for PEVK21.
- Based on the wormlike chain (WLC) model, we calculated the apparent persistence length (L_p) of peptides, which is a measure of bending rigidity. Under nondenaturing conditions L_p values were 0.63 and 0.48 nm for PEVK11 and PEVK21, respectively. The considerably different L_p values observed in case of the two peptides suggest that the peptides do not behave as ideal polymer chains, and the assumptions of the WLC model are likely not valid in their case. This means varying persistence length and/or intrachain interactions may be present within the PEVK peptides.
- Chemical denaturation caused an increase in the end-to-end distance of the peptides, caused most likely by the weakening of hydrophobic interactions.
- Upon increasing the ionic strength the end-to-end distance of the peptides decreased. A possible explanation is that, electrostatic screening may result in the weakening of intramolecular repulsion, leading to chain contraction.
- Calcium had negligible effect on PEVK conformation.
- In our temperature-dependent measurements the greater overall slope of the f' versus temperature function seen in the case of PEVK21 indicates that this peptide has a greater overall protein matrix elasticity than PEVK11.
- The molecular dynamics simulations indicate that PEVK21 tends to adopt a loose helical core structure, whereas PEVK11 fluctuates without well-defined secondary structure.
- In case of single-molecule force spectroscopy experiments the effective persistence length of the PPAK fragment was 0.68 (±0.27) nm, which is comparable to the persistence length values obtained from FRET measurements.

In the other part of our work, we investigated the effect of the C-terminal region of the *Drosophila* protein DAAM on the organisation of the actin filament system.

- In the polymerisation assays the CDAAM construct further increased the assembly rate of actin compared to the FH1-FH2 construct alone.
- The nucleation activity of the FH2 domain was abolished by the I732A mutation, which confirmed the previous *in vivo* observations.
- According to our total internal reflection fluorescence microscopy experiments the DAD domain of DAAM has no effect on the elongation of actin filaments, whereas the CDAAM inhibits the elongation to a lesser extent compared to the FH1-FH2 construct.
- The DAD/DAD+C constructs did not affect the polymerisation kinetics, but the increase of anisotropy values indicates that they can bind to actin monomers.
- In case of the DAD+C construct the C-terminally located amino acids play a critical role in the binding of actin monomers.

DISCUSSION

According to our results it is conceivable that the random, structureless unit of PEVK is ~10 amino-acids-long, but transient interactions such as electrostatic and, importantly, hydrophobic interactions, may arise in longer segments. Such weak interactions arise as enthalpic contributions to the elasticity of PEVK, which has been suggested previously by Linke *et al.* [6]. Considering that these weak interactions are modulated by external, solvent factors, titin may dynamically vary its apparent elasticity in response to rapid environmental changes. The two peptides used belong to the polyE sequence motifs, which represent only a smaller fraction of the whole PEVK segment. However, because there is no major difference in the amino-acid composition of our peptides and that of the overall PEVK, each containing amino acids that may participate in electrostatic or hydrophobic interactions, we find possible that our results may be extrapolated to other PEVK fragments or even the whole PEVK region.

Our fluorescence spectroscopy results demonstrate that the C terminal region of formin DAAM plays an important role in the formation of actin-formin interaction., whereas the polymerisation rate of pyrene-labelled actin did not change in the presence of the DAD construct. Nevertheless, the construct containing both DAD and FH2 domains significantly accelerated the polymerisation. Interestingly, the association rate was not affected by the mutant FH1-FH2 protein. Consequently, the abolishment of the nucleation activity of the FH2 domain by the I732A mutation, an effect previously described *in vivo*, was also confirmed in our *in vitro* results.

TIRF microscopy has been used to show that the elongation of actin is inhibited to a lesser extent in case of the construct which also contains the DAD domain in addition to FH2. Based on our results the DAD+C/DAD domains of DAAM did not influence the elongation. Thus, their actin monomer binding property does not affect this phase of the polymerisation.

The results obtained from steady-state fluorescence anisotropy measurements imply that the autoinhibitorydomain of *Drosophila* DAAM is able to bind actin monomers, however, the amino acids located C-terminally from the DAD domain play an important role in the binding.

All in all, our results suggest that the C-terminal parts of formin proteins exhibit unique properties. The small functional differences within the members of formin families and their heterogeneous effect on filament dynamics may account for the diversity and tissue-specific localization of formin proteins.

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LIST OF PUBLICATIONS

The publication the thesis is based on

Huber, T., Grama, L., Hetényi, C., Schay, G., Fülöp, L., Penke, B., Kellermayer, M.S.Z. (2012). Conformational dynamics of titin PEVK explored with FRET spectroscopy. Biophysical Journal, 103 (7), pp. 1480-1489. IF: 3.668.

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1. Grama, L., Nagy, A., Scholl, C., **Huber, T.**, Kellermayer, M.S.Z. (2005). *Local variability in the mechanics of titin's tandem Ig segments.* Croat. Chem. Acta 78, 405-411. IF: 0.936.
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1. **Huber, T.**, Szatmári, D., Mártonfalvi, Zs., Kellermayer, M.S.Z. *A titin PEVK domén szekvenciamotívumainak szerkezete és mechanikája.* 37. Membrán-Transzport Konferencia, Sümeg, May 22-25, 2007.
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12. **Huber, T.,** Majoros, A., Mihály, J., Migh, E., Nyitrai, M., Bugyi, B. *A DAAM formin autoregulációs doménjének aktin kötésben betöltött szerepe.* 44. Membrán-Transzport Konferencia, Sümeg, May 20-23, 2014.
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