PhD thesis

Conformational dynamics and functional characterization of the C-terminal tail of myosin 16

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INTRODUCTION

Myosins form a large and versatile superfamily of actin-based motor proteins that convert the chemical energy of ATP hydrolysis into mechanical force required for motion along actin filaments [1]. A novel unconventional myosin was described in 2001 by Patel et al., which was originally named as myr8 (8th unconventional myosin from rat) but later was designated as a new class: myosin XVI [2]. Two myosin 16 (Myo16) splice variants have evolved: Myo16a, the shorter, cytoplasmic isoform and Myo16b, the predominant, longer isoform having an additional 590 amino acid extension on its C-terminus. The unconventional Myo16 has an N-terminal pre-motor extension, called ankyrin domain composed of eight ankyrin repeats (Myo16Ank), followed by a conserved motor domain, an IQ motif in the neck region and a unique C-terminal tail extension in Myo16b isoform (Myo16Tail). The tail extensions are highly diverse among myosin classes both from structural and functional aspects. Some myosins are monomers containing functional domains, e.g. Src homology 3 (SH3), GTPase-activating protein (GAP), four-point-one, ezrin, radixin, moesin (FERM) or pleckstrin homology (PH) domains on their tail [3]. This structural diversity can lead to numerous intracellular activities and functions, such as cargo binding, dimerization, cellular localization, anchoring-tethering, protein-protein interaction, kinase activity or autoregulation [4–7].

In mammals, Myo16 is expressed predominantly in the embryonic and adult brain peaking during the 1-2 postnatal weeks in rats, and in a lesser amount, it can be found in some peripheral tissues. The expression of Myo16 coincides with neuronal cell migration, axonal extension and dendritic elaboration [2]. The N-terminal Myo16Ank interacts with protein phosphatase 1 catalytic subunit (PP1c) and regulates its phosphatase activity [8]. PP1c is involved in the control of synaptic plasticity, mechanism of learning and memory [9]. Myo16 is a component of the neuronal phosphoinositide 3-kinase (PI3K) signaling pathway, in which it is phosphorylated by the Src family of tyrosine kinase Fyn at its C-terminal tail [10]. Phosphorylated Myo16Tail interacts with PI3K and the WAVE1 regulatory complex (WRC) simultaneously. Thereby, Myo16 can mediate actin cytoskeleton remodeling through the WRC-Arp2/3 complex [10]. In line with this, Myo16 downregulates actin dynamics at the postsynaptic side of dendritic spines of Purkinje cells. In addition, Myo16 is also implicated in the organization of presynaptic axon terminals of parallel fibers [11,12]. Comprehensively, Myo16 seems to be important in the regulation of the morphological and functional features of parallel fiber-Purkinje cell synapses. Nuclear localization of Myo16b was observed in mouse cerebellum (P23, 31) *in vivo* [2,13], which is attributed to the C-terminal tail. Although this extension does not contain any typical nuclear localization sequence

(NLS). The motor domain function of Myo16 has not been described yet. The above findings suggest that Myo16 has a crucial role in different aspects of neuronal functioning. In addition, genetic alterations of *MYO16* were found to be involved in neurodegenerative disorders, including schizophrenia, autism spectrum disorder (ASD), bipolar disorder subtype II and major depressive disorder [14–16] which underlines its important role in the proper functioning of the nervous system.

Myo16b has a multi-faceted C-terminal extension consisting of a WAVE1 interacting region (WIR), a neuronal tyrosine-phosphorylated adaptor for phosphoinositide 3-kinase (NYAP) homology motif (NHM) [10], a proline-rich region (Pro-rich) and a distal C-terminal sequence element, which is presumed to be responsible for the nuclear localization of the protein through an atypical way [13]. Besides the diversity in functional motifs, the C-terminus of Myo16 is supposed to have a disordered structure [17]. Disordered proteins and disordered regions lack a unique 3D structure in their native, functional state [18–20], therefore these proteins or regions were named as intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs), respectively [20]. IDPs can be classified by the level of the structural disorder, such as molten globule, pre-molten globule and random coil [18]. The main attributions of IDPs and IDRs are the irregular amino acid composition resulting in high net charge accompanied by electrostatic repulsion and low hydrophobicity, which precludes the formation of globular structure [21]. The propensity of post-translational modifications (PTMs) is an important property of IDPs, which requires site accessibility, therefore PTM sites are located particularly in disordered regions providing a relatively large surface on the protein. IDPs have been suggested to be enriched in phosphorylation sites [22]. These structural properties have functional advantages in IDPs and IDRs attributing structural flexibility and thus plasticity to adapt to contextual changes provided by low affinity and high specificity of binding as well as interaction with extended partners or environmental factors [18,23–25]. Some of these functions have been already described in connection to Myo16b [10,13].

Here, we focused on the C-terminus of Myo16 (Myo16Tail) to characterize its conformational dynamics, structural properties and functions with the combination of sequence-based prediction, fluorescence and circular dichroism spectroscopy, as well as calorimetric approaches.

AIMS OF THE THESIS

The central interest of my PhD work was to investigate the structural properties, conformational dynamics and functional characterization of the recombinant C-terminal tail extension of non-conventional myosin 16 with the combination of sequence-based predictions, fluorescence-, circular dichroism spectroscopy and calorimetric methods.

Based on the amino acid sequence composition, the Myo16Tail might possesses intrinsically disordered structure under native conditions. In order to understand how Myo16Tail functions in biological processes, first the structural behavior was studied under *in silico* and *in vitro* circumstances.

The following questions were addressed:

- Does Myo16Tail possesses disordered structure based on bioinformatics analysis?
- How does the cooperativity and conformation of Myo16Tail influenced by the denaturant-induced unfolding?
- Does Myo16Tail contains secondary structural elements?
- Does Myo16Tail thermodynamically stable?

In the second part of my PhD work, the interactions of recombinantly produced Myo16Tail and Myo16Tail (-IQ) were investigated with fluorescently labeled Myo16Ank, Myo16IQ, profilin and actin by using *in vitro* biochemical and biophysical methods.

The following questions were addressed:

- Does the C-terminal Myo16Tail interacts with the N-terminal Myo16Ank?
- Does the IQ motif of Myo16 influence the binding properties of Myo16Tail?
- Does My16Tail regulates the assembly of the actin filaments?

MATERIALS AND METHODS

Bioinformatics

Disordered probability prediction for Myo16Tail was performed using VLXT [26],VL3-BA [27], VSL2b [28], Ronn [29] and IUPred servers [30]. Structural flexibility was analyzed by DynaMine server [31]. Phosphorylation site prediction was assessed using PhosphositePlus [32]. Multiple sequence alignment of Myo16Tail was performed by using Clustal X [33]. The 3D structural model was created by using I-TASSER [34].

Protein expression and purification

The DNA sequence of Myo16Tail (1146-1912 aa) containing the IQ motif and Myo16Tail (-IQ) (1176-1912 aa) without the IQ were cloned into pFastBac plasmid (ThermoFisher Scientific) possessing a His_6 affinity tag at the N-terminus of the constructs. Recombinant His_6-Myo16Tail consrtucts were expressed in Baculovirus/Sf9 system and purified by using Ni-NTA resin under denaturing conditions followed by renaturation. The expressed and purified Myo16Tail fragments were confirmed by using Western blot analysis. The N-terminal ankyrin domain of Myo16 (Myo16Ank) containing a gluthation S-Transferase (GST)-tag was expressed in *E. coli* (ER 25.66) and purified by using GST affinity resin [8]. Actin was purified from acetone-dried rabbit skeletal muscle powder according to standard protocols [35]. Myo16IQ (1146-1175) was synthesized by company (Genscript, Piscataway, NJ, US). Recombinant mouse profilin1 (profilin) was purified as described previously [36]. Myo16Ank, Myo16IQ and profilin were labeled by Alexa Fluor® C5 568 maleimide (Alexa568, Invitrogen), while actin was labeled by pyrene (Sigma-Aldrich).

Fluorescence spectroscopy experiments

Steady-state fluorescence emission measurements of tryptophan side-chains and 1-anilino-naphthalene-8 sulfonic acid (ANS) were monitored by Horiba Jobin Yvon Fluorolog 3.22 spectrofluorimeter using 5-5 μM of G-actin and Myo16Tail. Tryptophans were excited at 295 nm and the emission spectra were recorded between 300-450 nm, while ANS (250 μM) was excited at 360 nm and the emission was monitored from 400-650 nm using 2.5-2.5 nm slit in the absence and presence of increasing concentrations of GuHCl at 20 °C. The tryptophan fluorescence emission spectra of proteins were corrected by subtracting the GuHCl background intensity. To evaluate the cooperativity of unfolding we used Origin 2020 software and applied a sigmoidal function [37] to fit the maximum wavelength data of tryptophan and ANS fluorescence emission. In case of the ANS fluorescence of G-actin control Gaussian function was fitted.

Fluorescence quenching measurements of the tryptophans of G-actin and Myo16Tail was measured by using acrylamide quencher. The steady-state fluorescence quenching was obtained using Horiba Jobin Yvon Fluorolog 3.22 spectrofluorimeter. The fluorescence lifetime quenching was measured with Horiba Jobin Yvon Nanolog spectrofluorimeter (Horiba Scientific, Kyoto, Japan). The fluorescence intensities of tryptophans were determined at 350 nm for the Stern-Volmer analysis. Data were corrected with the inner filter effect during the analysis. Origin 2020 software was used for data evaluation using the Stern-Volmer model for steady-state fluorescence quenching and for lifetime quenching [38].

Steady-state fluorescence anisotropy of tryptophan side-chains were performed on the samples by adding the aforementioned series of GuHCl concentration. Moreover, the steady-state fluorescence anisotropy of Alexa568–Myo16Ank (1 μM and 1.2 μM) and Alexa568–Myo16IQ (1 μM) were measured to study the interaction of Myo16Tail and Myo16Tail (-IQ) with Myo16Ank, and Myo16Ank to Myo16IQ. The measurements were performed using Horiba Jobin Yvon Fluorolog 3.22 spectrofluorimeter (Horiba Scientific, Kyoto, Japan) (tryptophan, λ_{ex}=295 nm, λ_{em}=350 nm; Alexa568–Myo16Ank and Alexa568–Myo16IQ, λ_{ex}=578 nm, λ_{em}=601 nm). The anisotropy data was evaluated with the quadratic binding equation, where the dissociation equilibrium constants K_D were determined.

Time-Correlated Single-Photon Counting (TCSPC) fluorescence lifetime and anisotropy of tryptophans were measured by the means of TCSPC using a Horiba Jobin Yvon Nanolog spectrofluorimeter. The excitation was done by a 295 nm Nanoled as light source (Horiba Scientific, Kyoto, Japan), the fluorescence emission was measured at 350 nm. Time-resolved lifetime and anisotropy decay measurements were performed with 5-5 μM of G-actin and Myo16Tail by adding GuHCl. Time-resolved fluorescence lifetimes (τ) were calculated. Data were analyzed with Exponential Model [Reconvolution] by Fluofit software (PicoQuant, Berlin, Germany). Time-resolved anisotropy data were fitted by Origin 2020 software using two-exponential fit and rotational correlation (θ) values were derived.

Circular dichroism (CD) spectroscopy

Far UV (190-250 nm) CD spectrum of Myo16Tail was recorded at 25 °C using a Jasco J-810 spectropolarimeter (JASCO, Tokyo, Japan). The applied concentration of Myo16Tail was 12.7 μM (1.1 mg/ml) measured by the absorbance at 280 nm. The experimental parameters were: path length 0.01 cm, bandwidth 1 nm, scanning speed 20 nm/min, response time 4 s and 6 scans were accumulated. The far-UV CD spectrum was corrected for the baseline by subtracting the CD spectrum of the buffer measured under the same conditions. The CD spectra were analyzed by

the BeStSel [39,40] webserver for secondary structure composition. Thermal denaturation experiments were carried out at a concentration of 1.15 μM (0.1 mg/ml) in a 1 mm cell. Spectra were recorded in the temperature range of 10-100 °C with 10 °C steps accumulating 3 scans and using a heating rate of 1 °C/min between temperature points of spectrum collection.

Differential scanning calorimetry (DSC)

Thermal denaturation of 1 mg/ml G-actin (control) and Myo16Tail was measured by using Setaram Micro DSC-III. The measurements were carried out with a heating rate of 0.3 and 0.1 K/min in the temperature range of 20-100ºC, respectively. The buffer solutions of the protein of interests were used for reference measurements, respectively. At each measurements the protein samples were heated up consecutively twice in the aforementioned temperature range in order to confirm that G-actin and Myo16Tail irreversibly denatured during the 1st thermal denaturation process.

Polymerization kinetics

Polymerization of G-actin (2 μM, containing 2% pyrenyl-actin) was measured in the presence of Myo16Tail and profilin with Safas Xenius FLX spectrofluorimeter. The polymerization reaction was initiated by the addition of 100 mM KCl and 2 mM $MgCl_2$. Actin polymerization was measured by monitoring the increase in the pyrenyl fluorescence emission (λ_{ex} = 365 nm, λ_{em} = 407 nm). The polymerization velocity of actin was derived from the initial slope (0-500 s) of the pyrenyl fluorescence traces.

RESULTS AND DISCUSSION

Sequence-based *in silico* analysis of Myo16Tail predicts disordered regions

To begin the characterization of the conformational properties of rat Myo16Tail containing the IQ motif, its primary sequence was analyzed. The analysis revealed that the sequence is dominated by disorder-promoting amino acids contributing by 68% to the total number of residues, while the order-promoting amino acids are present at only around 32%.

Disorder prediction of Myo16Tail was performed with the combination of disorder predictors, including VLXT, VL3-BA, VSL2b, Ronn and IUPred. The C-terminally located regions are characterized by a relatively high disorder probability.

For additional confirmation, we analyzed the disorder probability of sequences of different vertebrate representatives of Myo16Tail using IUPred: *Homo sapiens (Hs*), *Mus musculus (Mm)*, *Rattus norvegicus (Rn)*, *Gallus gallus (Gg)*, *Xenopus tropicalis (Xt*), *Danio rerio (Dr)*. Based on the IUPred prediction the sequences of representatives display similarly high disorder probability suggesting that the intrinsically disordered structure is conserved in Myo16Tail. In line with this, we analyzed the conservation of Myo16Tail sequence from different vertebrate classes in multiple sequence alignment by using Clustal X [33]. The analyses showed substantial amount of conservation of Myo16Tail in the IQ and NHM motifs, but not in the proline-rich region. In addition, other regions also display considerable amount of conservations, which suppose that the predicted disordered segments are mostly conserved suggesting that the intrinsically disordered nature of Myo16Tail is conserved through the evolution. Since Myo16Tail might be involved in neurodegenerative disorders [14–16], the conserved nature of Myo16Tail structure can have significance in the functioning of the human Myo16.

Myo16Tail sequence was further assessed with DynaMine to characterize the protein backbone flexibility. According to the DynaMine prediction, ~70% of Myo16Tail shows high backbone flexibility (S^2 values < 0.7) and only ~20% of Myo16Tail has rigid structure (S^2 values > 0.8). There is a context-dependent range (10%) where the protein of interest might be able to switch between conformational states or folding can occur toward a more ordered structure upon binding to a partner molecule.

As intrinsically disordered regions (IDRs) are enriched in post-translational modification sites, the PTMs of Myo16Tail were analyzed using PhosphositePlus [32]. The prediction showed that several phosphorylation sites might occur in Myo16Tail based on references (11 Ser, 4 Thr, 3 Tyr phosphorylation sites). To assess the relevance of these PTMs, the conservation of predicted Myo16Tail phosphosites was analyzed in the aforementioned multiple sequence

alignment. Three out of the predicted phosphorylation sites are located in the NHM motif and showed to be fully conserved from fish to human. Two of them (Tyr^{1416} and Tyr^{1441}) were identified to be phosphorylated by Fyn kinase that eventuates the recruitment of the p85 subunit of the phosphoinositide 3-kinase and the activation of PI3K signaling pathway [10]. The rest of phosphosites can be found particularly in the disordered regions, notably 10 out of 14 phosphosites are fully conserved and one of them is weakly similar. The conservation of phosphosites suggests that phosphorylations in the disordered regions of Myo16Tail might have particular importance in the evolution of vertebrate Myo16.

Since structural data have not been available for Myo16Tail yet, we created a 3D structural model of Myo16Tail by using Iterative Threading Assembly Refinement (I-TASSER). The 3D model of Myo16Tail predicts α-helical, β-sheet and turn components besides the considerable amount of disordered structural elements supposing a molten globule-like behavior of Myo16Tail. The molten globule state of a protein is less compact, more flexible and dynamic than a globular fold [41].

Myo16Tail shows low cooperativity of unfolding

In order to investigate the structural properties of Myo16Tail experimentally, we performed steady-state fluorescence measurements by using its tryptophan residues as intrinsic probes as well as ANS fluorescence assays. Myo16Tail contains six tryptophan residues, three of them are located in more ordered segments, whereas the other three are in the predicted disordered regions. In line with this, the position of tryptophan residues in Myo16Tail structural model shows similar distribution. Tryptophans, as intrinsic fluorophores are highly sensitive to their local environment [42]. When tryptophans are exposed to solvent, the maximum wavelength is around 350 nm [43] or even can be further red-shifted towards 360 nm [38]. In order to test the results of bioinformatic analysis at first, the tryptophan fluorescence emission of Myo16Tail was measured to reveal the unfolding properties in the presence of increasing concentrations of GuHCl as a denaturing agent. Our preassumption was that unfolding of the protein (thus the local environment of tryptophans) should result in a red shift.

Myo16Tail exhibited a red-shifted maximum wavelength around 350 nm, even in the absence of denaturant suggesting that tryptophan side-chains are already solvent accessible in the native molecule. The decrease of fluorescence intensity of Myo16Tail upon titration by GuHCl was smaller as compared to that of observed for G-actin, as well as the observed red-shift was less pronounced, from 351 to 360 nm. These observations indicate that tryptophans of Myo16Tail are highly accessible suggesting that they are located in a less-structured, disordered protein

matrix. The GuHCl concentration dependence of the maximum wavelength further supported the different conformational characteristics of the two proteins. The pronounced and steep sigmoid tendency observed for G-actin is indicative of cooperative conformational transitions upon unfolding. In contrast, the trend detected for Myo16Tail suggests modest or the lack of cooperativity. Cooperativity of protein folding/unfolding denotes the changes of secondary and tertiary interactions; i.e. high cooperativity correlates with ordered, globular fold, while low cooperativity indicates disordered structure [44,45].

To study the structural behavior of Myo16Tail in more detail, 1-anilino-naphthalene-8 sulfonic acid (ANS) fluorescence measurements were performed. ANS is a hydrophobic fluorescent probe which is used to reveal the hydrophobic sites and characterize the molten globule conformation of proteins [46]. ANS does not bind to well-ordered proteins, either to totally unfolded ones, but it displays maximum intensity when molten globule conformation can occur [46]. Myo16Tail showed a relatively high ANS fluorescence intensity already in the absence of GuHCl that decreased upon GuHCl addition in a sigmoidal manner with a less steep transitions as compared to that observed for G-actin. Importantly, the highest intensity and wavelength maximum of ANS fluorescence of Myo16Tail at low GuHCl (~480 nm) is in accordance with the maximum values of G-actin in the range of 1-2 M GuHCl (~480 nm), when the latter one is in molten globule conformation. Our ANS fluorescence results corroborate that Myo16Tail might have molten globule conformation under native conditions.

To corroborate the above conclusions, tryptophan fluorescence quenching was performed. Quenching of tryptophan residues of Myo16Tail and G-actin by acrylamide was monitored in steady-state fluorescence emission measurements. Classical Stern-Volmer plot of steady-state fluorescence quenching of tryptophans showed increased K_{sv} values for Myo16Tail (K_{sv} = 2.59 ± 0.07 M^{-1}) as compared to that of characteristic to G-actin (K_{sv} = 1.98 ± 0.01 M^{-1}) suggesting higher tryptophan accessibility and conformational dynamics of Myo16Tail. As one can observe, the Stern-Volmer plot of Myo16Tail deviates from linear. This suggests that the quenching mechanism cannot be described only by collisional quenching. An upward curvature was observed in those cases when the fluorophores form a so-called dark complex with the quencher and thus static quenching takes place [47]. As the dark complexes cannot be excited, they will not contribute to the measured fluorescence lifetime in the time-resolved measurements.

In the case of fluorescence quenching measurements observed by measuring the fluorescence lifetime, the formation of dark complexes will have no effect on the fluorescence lifetime. If only static quenching – and dark complex formation – happens the τ_0/τ Stern-Volmer plot will be a flat line. To check the contribution of static quenching, we performed acrylamide

quenching experiments by measuring the fluorescence lifetimes using time-correlated single-photon counting. The Stern-Volmer plot of G-actin is linear, and we can conclude that the quenching is collisional. Despite, the Stern-Volmer plot of measurements on Myo16Tail shows a moderate increase until 0.5 M acrylamide after that it is relatively flat. This suggests that in the case of Myo16Tail there is a significant static quenching part aside from the collisional quenching. Observing the average fluorescence lifetime of tryptophans of Myo16Tail one can see that the lifetime drops significantly – from 4.1 ns to 3.0 ns – by adding ~0.5 M acrylamide. Above this concentration the increasing amount of acrylamide has only minor effect on the average lifetime which levels of around 2.8 ns. In comparison the quencher has a stronger effect in the case of G-actin: as one can observe, the average lifetime of tryptophans decreases linearly with increasing quencher concentration. In the time-resolved quenching measurements, the increasing quencher concentration results in decreasing fluorescence lifetime of Myo16Tail, which means that above ~0.5 M the acrylamide forms dark complex with the tryptophans. The possible explanation of this finding is that the solvent-exposed tryptophans are quenched efficiently by acrylamide.

Conformation dynamics of Myo16Tail

Steady-state tryptophan fluorescence anisotropy of G-actin and Myo16Tail was compared upon chemical denaturation induced by GuHCl. A slight sigmoidal response was detected for Myo16Tail upon addition of GuHCl, also the low steepness of the transition curve suggests low cooperativity in unfolding [48], in agreement with the fluorescence emission measurements and with the ANS fluorescence findings.

Time-correlated single-photon counting data showed that the decrease in tryptophan lifetime correlates upon denaturation by increasing concentration of GuHCl. The average lifetime of tryptophan residues of control G-actin Myo16Tail revealed a similar, slightly sigmoidal decline upon chemical denaturation as we observed in steady-state anisotropy measurements. The time-resolved anisotropy decay of G-actin with a molecular mass of 42 kDa showed a rotational correlation time of ~26 ns and decreasing with increasing GuHCl concentrations. The reported values show the rotation of the whole protein. Decreasing rotational correlation times reflect the increased mobility of the segments having the tryptophans.

The time-resolved anisotropy of Myo16Tail in the absence of GuHCl revealed a two exponential decay; a faster ~1 ns and a longer ~33 ns phase. The fast phase can be assigned as the standalone rotation of tryptophan residues. The longer phase detected for Myo16Tail is practically equal to the expected value for a protein using the experimental formula by Visser [49] with the corresponding molecular weight (~86.5 kDa). This result shows that in Myo16Tail, there

is an ensemble of tryptophans, which are at least partially unburied and they are more solvent-exposed. The rotation of these solvent-exposed tryptophans is less restricted that is the reason of the presence of the ~1 ns component in the rotational correlation times.

These findings agree with the correlation between the locations of tryptophan residues in the predicted structural model and the disorder probability of the corresponding regions. Both, the steady-state and time-correlated fluorescence results indicate that Myo16Tail can undergo slight conformational transitions upon unfolding suggesting that the disordered regions are associated with some ordered structural elements.

Secondary structure analysis of Myo16Tail by CD spectroscopy reveals structured regions

The CD spectrum of Myo16Tail in the far-UV region revealed the minimum at 205 nm, the positive maximum at 190 nm and a significant signal in a wide, 215-225 nm region. This suggests that the protein contains both α-helical and β-structured elements. On the other hand, the large minimum at 205 nm and the relatively weak positive maximum around 190 nm indicate the presence of a significant amount of disordered structure, as well. The CD-spectroscopic analysis of G-actin (control) that was described and published earlier revealed two major negative minima in the far-UV region at 211 nm and 221 nm, suggesting significantly higher amount of α-helical and β-structured elements as compared to what we detected in Myo16Tail [50].
Beta Structure Selection method (BeStSel) was used as a deconvolution algorithm to assess CD data. BeStSel analysis showed that the secondary structure content of Myo16Tail appeared to be due to 19.5% α-helix and 21.3% β-sheet, where the contributions of antiparallel β subclasses are 0.0% anti1 (left-twisted), 5.4% anti2 (relaxed) and 15.9% anti3 (right-twisted). Further structures in form of 15.2% turn and 44.0% “others” are present, the latter accounts mainly for the disordered content. These findings are in line with the disorder prediction, in which some segments are predicted to be ordered. Altogether, the turn and disordered structure make up at least 60% in Myo16Tail, which is consistent with the primary amino acid sequence-based bioinformatic findings. The mean residue ellipticity of far-UV CD signals of Myo16Tail were analyzed on a double wavelength plot, $[\theta]_{222}$ against $[\theta]_{200}$, provided by Uversky and Fink [51] in order to classify Myo16Tail as a molten globule or pre-molten globule conformation. Based on the plot Myo16Tail can be found between the molten and pre-molten globule populations.

Thermal denaturation of Myo16Tail was carried out by heating up the protein from 10 °C to 100 °C and recording the CD spectra at 10 °C steps. The spectra gradually changed upon increasing the temperature, although Myo16Tail showed only minor spectral changes without a well-defined unfolding transition. At higher temperatures, some precipitation of the protein was

observable, however, even with precipitation, there were only minor spectral changes. The secondary structure analysis showed a ~7% decrease in the α-helix content and ~5% increase in the disordered content. The β-structure content is increased by ~4%, which could be the result of partial aggregation of the protein at high temperatures. The thermal behavior of Myo16Tail showing low cooperativity detected by CD measurements supports the idea of lacking a stable globular fold and is characteristic of a molten globule-like state.

Myo16Tail is thermodynamically unstable

The thermodynamic stability of Myo16Tail was studied by using differential scanning calorimetry (DSC) at different heating rate. Disordered proteins lack of endotherm cooperative denaturation and oligomerization and/or aggregation can occur due to the solvent-exposed hydrophobic groups representing an exotherm reaction [52] or exotherm cooperative folding [53]. At first, 1mg/ml Myo16Tail was thermally denatured at a heating rate of 0.3 K/min between 20-100 °C. The analysis showed that Myo16Tail lack of a well-defined cooperative denaturation compared to G-actin control and tend to aggregate above ~50 °C. The noncooperative endothermic transition of Myo16Tail might occurs due to the fast aggregation followed by melting of the aggregates. The calorimetric measurements were repeated by using 0.1 K/min heating rate between 20-100 °C. The analysis showed an exotherm cooperative transition between 45-80 °C suggesting that Myo16Tail probably undergoes aggregation which is manifested as an exotherm cooperative folding due to the strengthening of the hydrophobic interactions caused by the slow temperature elevation.

Myo16Tail is functionally active and interacts with Myo16Ank

To study the functional activity of Myo16Tail we aimed to characterize its possible binding properties. We performed binding assay using steady-state fluorescence anisotropy measurements to test the interaction between Myo16Tail with the N-terminal Myo16Ank region. The steady-state anisotropy of fluorescently labeled Alexa568–Myo16Ank (1.2 μM) increased by the addition of increasing concentration of Myo16Tail as expected for binding interaction. The analysis revealed that the affinity (K_D) of Myo16Tail to Myo16Ank is ~2.5 μM.

In order to characterize the binding properties of the tail of Myo16 in more detail, we measured the steady-state anisotropy of Alexa568–Myo16Ank (1 μM) in the presence of increasing concentration of a new recombinant Myo16Tail without the IQ motif, Myo16Tail (-IQ). The fit to the anisotropy data resulted in weaker affinity with ~5.6 μM dissociation equilibrium constant. Moreover, to investigate the binding contribution of unoccupied IQ motif to Myo16Ank,

we measured the steady-state anisotropy of Alexa568-labeled, synthesized rat Myo16IQ in the presence of increasing concentration of Myo16Ank. The steady-state anisotropy of Alexa568–Myo16IQ revealed that Myo16Ank is able to interact with Myo16IQ albeit, with much weaker affinity (K_D = ~16 μM) to that of both, Myo16Ank:Myo16Tail and Myo16Ank:Myo16Tail (-IQ) complexes.

Altogether, in one hand our results can confirm the functional activity of the recombinantly produced Myo16Tail and Myo16Tail (-IQ). On the other hand, our anisotropy findings revealed that the tail of Myo16 is dominant in the binding of Myo16Ank, however, the presence of Myo16IQ seems to influence the strength of this interaction.

Myo16Tail does not influence the actin polymerization dynamics

In order to study the possible interaction between prolin-rich motif containing Myo16Tail and profilin, steady-state fluorescence anisotropy of Alexa568–profilin was performed in the presence of increasing concentration of Myo16Tail. The results showed no change in anisotropy suggesting that Myo16Tail does not bind to profilin through the prolin-rich motif. Moreover, we investigated the profilin–G-actin complex hypothesizing that during the complex formation a favourable conformation might occurred for binding of Myo16Tail. The steady-state anisotropy of Alexa568–profilin in complex with G-actin does not increased when Myo16Tail was added in maximal concentration (15 μM) ($p>0.05$) further suggesting that there is no interaction between Myo16Tail and profilin.

The polymerization of pyrene-labeled G-actin (2 μM) was measured in the presence and absence of Myo16Tail and profilin. For the quantitative analysis, the polyimerzation rate was derived from the slope of the initial phase (0-500 s) of the normalized polymerization curves. The actin polymerization assays revealed that Myo16Tail does not able to influence the polymerization dynamics of actin in the absence and presence of profilin. Although, the neuronal-specific profilin isoform (profiling 2) might be able to interact with Myo16Tail

Comprehensively, our bioinformatic and conformational findings suggest the disordered, molten globule-like nature of Myo16Tail. These structural data are in line with our functional results according to which the C-terminal Myo16Tail is able to interact with the N-terminal Myo16Ank through a flexible backfolding and might be playing a role in autoregulation of the Myo16 motor domain.

SUMMARY

The tail extension in Myo16b isoform is involved in the neuronal PI3K signaling pathway and supposed to have intrinsically disordered structure. However, its detailed biological functions and structural features remained unclear. Here, a combination of bioinformatics, spectroscopic and calorimetric methods were used to investigate the conformation dynamics, structural properties and the functional behavior of Myo16Tail.

- Disorder probability bioinformatic analysis showed that Myo16Tail has considerable amount of flexible, disordered regions in its structure.
- PhosphositePlus predicts conserved phosphorylation sites in the NHM and in the disordered regions of Myo16Tail and the multiple sequence alignment suggests high conservation of the disordered segments of Myo16Tail.
- The structural model supports the intrinsically disordered nature of Myo16Tail.
- Myo16Tail shows weak cooperative unfolding.
- Tryptophans of Myo16Tail are more accessible (K_{SV}=2.59 ± 0.01 M^{-1}) and static quenching is dominant compared to G-actin control (K_{SV}=1.98 ± 0.01 M^{-1}).
- Denaturant-induced unfolding of Myo16Tail revealed a multi-phase conformation dynamics, in which a fast (~1 ns), and a slow (~33 ns) components occur due to the rotation of tryptophans and the whole Myo16Tail, respectively.
- Myo16Tail shows a considerable amount of intrinsic disorder (44%) besides the secondary structural elements.
- Myo16Tail is thermodynamically unstable and undergoes thermally-induced exotherm cooperative folding presumably due to aggregation.
- Both, Myo16Tail or Myo16Tail (-IQ) binds the N-terminal Myo16Ank with a K_D of ~2.5 μM and ~5.6 μM in a similarly moderate range of affinity, respectively.
- Myo16Tail does not bind profilin and profilin–G-actin complex and does not influence the actin polymerization dynamics through its proline-rich region.

Myo16Tail might act as a dynamic molten globule and may function as a flexible display site [54]. The presence of disordered structural regions may help to understand how Myo16 behaves during interactions with binding partners (Myo16Tail interacts with PI3K and WAVE1, Myo16Ank), in phosphorylation processes or in signaling pathways playing crucial role in regulation [10]. Signal transduction and neurodegenerative diseases are associated with proteins possessing intrinsically disordered structure [55].

LIST OF PUBLICATIONS

Publications related to my PhD work

1. Elek Telek, Kristóf Karádi, József Kardos, András Kengyel, Zsuzsanna Fekete, Henriett Halász, Miklós Nyitrai, Beáta Bugyi, András Lukács. The C-terminal tail extension of myosin 16 acts as a molten globule, including intrinsically disordered regions, and interacts with the N-terminal Ankyrin. *Journal of Biological Chemistry*. 297(1) 100716. (2021) IF: 5.157
2. Elek Telek, András Kengyel, Beáta Bugyi. Myosin XVI in the nervous system. *Cells* (review), 9, E1903. (2020), IF: 4,366

All public citation: 3, independent citation: -

Conference talks related to my PhD work

1. Telek Elek, Karádi Kristóf, Kardos József, Kengyel András, Nyitrai Miklós, Bugyi Beáta, Lukács András. A miozin 16 C-terminális szerkezetének karakterizálása spektroszkópiai módszerekkel. *MBTF Molekuláris Biofizika szekció ülés*, miniszimpózium, Pécs, 2020. 01. 28.
2. Telek Elek, Kengyel András, Nyitrai Miklós. A miozin 16 C-terminális domén biokémiai karakterizálása. *Emberi Erőforrások Minisztériuma, Új Nemzeti Kiválóság Program konferencia*, Pécs, 2018. 05. 25.
3. Telek Elek, Holló Alexandra, Bécsi Bálint, Kengyel András, Erdődi Ferenc, Nyitrai Miklós. Binding Properties of the Disordered Myosin 16 Tail Domain. *7th Interdisciplinary Doctoral Conference*, Pécs, 2018. 05. 17-19.
4. Telek Elek, Kengyel András, Nyitrai Miklós. A miozin 16 C-terminális domén interakciói és funkciói. *Magyar Biofizikai Társaság XXVI. Kongresszusa*, Szeged, 2017. 08. 22-25.
5. Telek Elek, Kengyel András, Nyitra Miklós. Biochemical Characterization of Myosin 16 Tail domain. *5th Interdisciplinary Doctoral Conference*, Pécs, 2016. 05. 27-29.

Conference posters related to my PhD work

1. Telek Elek, Karádi Kristóf, Kengyel András, Kardos József, Beáta Bugyi, Nyitrai Miklós, Lukács András. A rendezetlen szerkezetű miozin 16 C-terminális szerkezeti karakterizálása. *Magyar Biofizikai Társaság XXVII. Kongresszusa*, Debrecen, 2019. 08. 26-29.
2. Telek Elek, Kengyel András, Holló Alexandra, Kónya Zoltán, Erdődi Ferenc, Nyitrai Miklós. Functional characterization of the disordered myosin 16 tail domain. *Current Trend sin*

Biomedicine: Actin-based Mechanosensation and Force Generation in Health and Disease workshop, Baeza, Spain, 2019. 11. 11-13.

3. Kengyel András, Telek Elek, Holló Alexandra, Nyitrai Miklós. Autoregulatory functions of myosin 16 domains. *47th European Muscle Conference*, Budapest, 2018. 08. 30 - 09. 03.

4. Telek Elek, Holló Alexandra, Bécsi Bálint, Kengyel András, Erdődi Ferenc, Nyitrai Miklós. A rendezetlen szerkezetű miozin 16 C-terminális domén interakciói. *48. Membrán-Transzport Konferencia*, Sümeg, 2018. 05. 15-18.

5. Telek Elek, Holló Alexandra, Bécsi Bálint, Kengyel András, Erdődi Ferenc, Nyitrai Miklós. Binding Properties of the Disordered Myosin 16 Tail domain. *62_{nd} Annual Meeting of the Biophysical Society*. San Francisco, CA, USA, 2018. 02. 17-21.

6. Kengyel András, Telek Elek, Kónya Zoltán, Bécsi Bálint, Erdődi Ferenc, Nyitrai Miklós. Biochemical Characterization of Myosin 16 Domains. *Hungarian Molecular Life Sciences*, Eger, 2017. 03. 30. - 04. 02.

7. Kengyel András, Telek Elek, Kónya Zoltán, Bécsi Bálint, Erdődi Ferenc, Nyitrai Miklós. Interactions and functions of Myosin 16 Domains. *61st Annual Meeting of the Biophysical Society*. New Orleans, LA, USA, 2017. 02. 11-15.

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