The effect of toxofilin on the structure and dynamics of monomeric actin

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Article info
Article history:
Received 16 March 2015
Revised 25 August 2015
Accepted 31 August 2015
Available online 5 September 2015
Edited by Michael R. Bubb

The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

Keywords:
Cytoskeleton
Actin
Toxofilin
Dynamic
Fluorescence
Calorimetry

Abstract
The effects of toxofilin (an actin binding protein of Toxoplasma gondii) on G-actin was studied with spectroscopy techniques. Fluorescence anisotropy measurements proved that G-actin and toxofilin interact with 2:1 stoichiometry. The affinity of toxofilin to actin was also determined with a fluorescence anisotropy assay. Fluorescence quenching experiments showed that the accessibility of the actin bound e-ATP decreased in the presence of toxofilin. The results can be explained by the shift of the nucleotide binding cleft into a closed conformational state. Differential scanning calorimetry measurements revealed that actin monomers become thermodynamically more stable due to the binding of toxofilin.

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1. Introduction

Structural and dynamic changes in the actin cytoskeleton play central role in many cellular processes [1]. The structure and dynamics of the actin cytoskeleton is regulated by a large number of actin binding proteins [2].

Toxoplasma gondii is an intracellular pathogen that can use the actin cytoskeleton of the invaded cells for its own motility. The invasion of the host cells is an active process [3]. Toxofilin has a molecular mass of 27 kDa and contains 245 amino acid residues. This actin binding protein has no known sequence similarities with other actin binding proteins [4]. It can bind to actin monomers (KD = 2.3 µM) and to the ends of the actin filaments as well [4,5]. Toxofilin is secreted by T. gondii tachyzoite. Toxofilin can accelerate the turnover of actin filaments and able to facilitate parasite invasion through the host cell’s actin cytoskeleton [3]. Three independent actin binding regions were identified in the protein [6]. It has contact points with the subdomain 4 of actin and with the bound nucleotide as well. The toxofilin forms a ternary complex with an antiparallel actin dimer and this complex can bind to the barbed end of the actin filaments [6]. In the presence of toxofilin the polymerisation rate of actin is decreased [4]. The secreted toxofilin can disintegrate the cytoskeleton of the host cell, increase the ratio of the non-polymerised actin by binding the antiparallel dimers and can compete for the actin monomers with other actin binding proteins [6]. The overexpression of the GFP tagged toxofilin reduced the number of microfilaments and actin bundles within HeLa cells [4]. It was proved that the secretion of toxofilin can occur during the T. gondii infection [7].

In the present work the effect of toxofilin on the structural dynamics of non-parasitic actin was characterised. During the measurements only the segment spanning the amino acids 69–196 of the original protein was used. The two main activities
of the toxofilin (i.e. the monomer sequestration and barbed-end filament capping) can be related to the applied fragment. Considering that the studied toxofilin<sub>69–196</sub> is only a part of the total it is reasonable to think that the conclusions can be further refined by studying the whole protein sequence. The results showed that the toxofilin can bind two actin monomers simultaneously. Toxofilin reduced the accessibility of the actin bound nucleotide, which indicates the significant rearrangement of the nucleotide binding cleft on actin. The decreased accessibility of the nucleotide binding region was accompanied by the increased thermal stability of the monomeric actin.

2. Materials and methods

2.1. Chemicals

KCl, CaCl<sub>2</sub>, TRIZMA Base (tris-(hydroxy-methyl) amino-methane), MgCl<sub>2</sub>, acrylamide, MOPS (3-(N-morpholino) propane-sulfonic acid), imidazole, DOWEX 1x2-400, 2X YT Microbial Medium EZMix<sup>™</sup> Powder, kanamycin, IPTG (isopropyl-β-D-thiogalactopyranoside) and thrombin were purchased from Sigma–Aldrich (Budapest, Hungary). The e-ATP (1,5N<sub>2</sub>-ethenoadenosine 5'-triphosphate) was obtained from Invitrogen (Carlsbad, USA).

2.2. Protein preparation and fluorescent labelling of actin

Actin was prepared from acetone-dried muscle powder from rabbit skeletal muscle [8] according to the method of Spudich and Watt modified by Mossakowska et al. [9,10]. The Ca<sup2+</sup>-G-actin was stored in buffer A (4 mM Tris, 0.2 mM ATP, 0.1 mM CaCl<sub>2</sub>, 0.5 mM MEA, 0.005% NaN<sub>3</sub>, pH 8.0). The concentration of the G-actin was determined by using the absorption coefficient of 1.11 mg ml<sup>−1</sup> cm<sup>−1</sup> at 280 nm [11]. All the experiments were completed in the presence of Ca<sup2+</sup>-G-actin.

The DNA fragment encoding toxofilin<sub>69–196</sub> was ligated into a pET28a plasmid. Escherichia coli BL21 (DE3) pLysS cells were transformed with a plasmid construct and grown in 2X YT Microbial Medium EZMix<sup>™</sup> Powder in the presence of 30 µg/ml kanamycin until the OD of the culture at 600 nm reached 0.6–0.8. Expression was induced by 1 mM IPTG and performed for overnight at 20 °C. The clarified cell lysates were loaded onto a nickel-nitrilotriacetic acid-agarose column (Qiagen, Hungary) and eluted with increasing imidazole concentration. His-tag removal was carried out by digestion with thrombin at 4 °C overnight. Toxofilin was concentrated to 2–3 ml (Vivaspin 10 kDa cut-off, Sartorius Stedim Biotech GmbH, Germany) and loaded onto a Superdex-75 HiLoad gel filtration column (GE Healthcare) to remove thrombin and the cleaved His-tag peptides. The toxofilin<sub>69–196</sub> (50 mM Tris (pH 7.3), 50 mM NaCl, 5 mM DTT, 3% sucrose) was concentrated to 20–70 µM. Protein concentration was determined by using the molar extinction coefficient of 4470 M<sup>−1</sup> cm<sup>−1</sup> (ProtParam).

The labelling of actin with e-ATP was performed as described previously [12–14].

2.3. Fluorescence measurements

Steady-state fluorescence anisotropy measurements were performed with a Horiba JobinYvon Fluorolog-3 fluorimeter (Longjumeau Cedex, France) through the tryptophan of actin at 22 °C. The excitation and emission wavelength was 295 and 334 nm, respectively. The affinity of the toxofilin for actin was determined by using the following equation [15–17]:

\[
\frac{s_{\text{actual}} - s_{\text{min}}}{s_{\text{max}} - s_{\text{min}}} = \left( \frac{[\text{Act}] + [\text{Tox}] + K_D}{[\text{Act}] + [\text{Tox}] + K_D} \right) - \sqrt{\left( [\text{Act}] + [\text{Tox}] + K_D \right)^2 - 4[\text{Act}][\text{Tox}] L_{\text{total}}} \]

(1)

where \( s_{\text{actual}} \) is the actually measured signal, \( s_{\text{min}} \) is the signal from the un-complexed protein, \( s_{\text{max}} \) is the highest signal from the complexed protein, \( L_{\text{total}} \) is the total ligand concentration, \( P_{\text{total}} \) is the total protein concentration and \( K_D \) is the equilibrium dissociation constant.

In case of the fluorescence anisotropy measurement the \( s_{\text{actual}} \) was measured anisotropy value (\( r \)) at a certain toxofilin concentration, \( s_{\text{min}} \) represents the anisotropy of the un-complexed actin monomer (\( r_{\text{min}} \)), and \( s_{\text{max}} \) is related to the highest anisotropy value (\( r_{\text{max}} \)) when all binding sites are occupied on the actin monomer.

With the fluorescence quenching experiments it is possible to test the conformational changes in actin in the presence of toxofilin. When more than one fluorophore population can be found in the sample a complex form of the Stern–Volmer equation has to be applied:

\[
F_0 = \left( \sum_{i=1}^{n} \frac{f_i}{1 + K_{SV_i} [Q]} \right)^{-1}
\]

(2)

where \( F_0 \) is the fluorescence intensity in the absence of quencher, \( F \) is the fluorescence intensity at different quencher concentration (\( Q \)) and \( K_{SV_i} \) and \( K_{SVn} \) are the static and dynamic Stern–Volmer constant of the \( i \)th population represented by the fraction of \( f \) [13]. The \( K_{SV} \) values give information about the accessibility of the fluorophore to the quencher molecules. A decrease in the \( K_{SV} \) value can suggest decreased accessibility due to a closed nucleotide binding.

Time-resolved fluorescence measurements were performed with an ISS K2 Multifrequency Phase Fluorometer (ISS Fluorescence Instrumentation, Champaign, IL, USA) at 22 °C.

2.4. Nucleotide exchange assay

The nucleotide exchange assay was completed with e-ATP labelled actin monomers in the absence and presence of toxofilin with a stopped-flow machine. We applied 2 µM Ca-G-actin labelled with e-ATP and toxofilin with a final concentration between 0 and 8 µM. To displace the bound e-ATP from actin the protein sample was mixed with 2 mM ATP so the final concentration of the Ca-G-actin and ATP in the sample was 1 µM and 1 mM, respectively. The fluorophore was excited at 320 nm and the fluorescence emission was filtered with an FG 385 cut-off filter.

2.5. Differential scanning calorimetry (DSC) experiments

The DSC experiments were carried out with a Setaram Micro DSC II calorimeter. The heat denaturation curves were recorded between 0 °C and 100 °C with 0.3 K/min scanning rate. The denaturation of 30 µM monomeric actin was measured without and also in the presence of 17 µM toxofilin. The heat flow was plotted against the temperature and was analysed by using the MicroCal Origin 6.0 software. The melting temperatures (\( T_m \)) were used as thermal denaturation parameters.

3. Results and discussion

In this work the effect of toxofilin on the structure and dynamics of actin was studied by fluorescence spectroscopy and DSC measurements. In the measurements the 128 amino acid long segment (toxofilin<sub>69–196</sub>) of toxofilin was used that can be related
to the actin monomer sequestering and barbed-end filament capping function of this actin binding protein [6].

3.1. Binding of toxofilin (69–196) to the monomeric actin

The binding stoichiometry of toxofilin to actin can be determined from fluorescence anisotropy measurements if the protein concentration in the experiments is much higher than the $K_D$ value [16]. As for the equilibrium dissociation constant 2.3 μM was suggested previously [5] a relatively high concentration of actin was used (20 μM) in these anisotropy measurements while the toxofilin concentration changed between 0 and 40 μM in 10 steps (Fig. 1A). Under the applied conditions most of the added toxofilin bind to the actin as the protein concentration is much higher than the $K_D$.

The measured steady-state anisotropy value as a function of toxofilin concentration follows a break-point titration curve and the concentration where the breaking is observed indicates how much toxofilin is needed to bind all the actin. The data showed (Fig. 1A) that approximately 10 μM toxofilin is saturated with the 20 μM actin which indicates that one toxofilin could bind two actin molecules. This result corroborated the previous conclusion from analytical ultracentrifugation that toxofilin can bind two actin monomers [6].

Using lower protein concentrations the affinity of toxofilin for actin can also be revealed in steady-state fluorescence anisotropy measurements by calculating the equilibrium dissociation constant ($K_D$) for the G-actin–toxofilin complex. The G-actin concentration was 2 μM during the measurements while the toxofilin concentration changed from 0 μM to 12 μM in six steps. The anisotropy changed from 0.098 to 0.124 with the increasing toxofilin concentration (Fig. 1B). In the presence of toxofilin the fluorescence intensity of actin did not change considerably.

The increase of the steady-state anisotropy is attributed to the binding of toxofilin to actin. The effect of toxofilin on the anisotropy of actin can be manifested through two basic modalities. Due to the formation of a large protein–protein complex the rotational diffusion of actin becomes slower. If this is the dominant effect then one would expect that the steady-state anisotropy is different for the hetero-dimer and hetero-trimer forms of the toxofilin–actin complexes.

An alternative explanation for the increased anisotropy is based on the local changes induced by the binding of toxofilin around the fluorophores on actin. The micro-environmental change can hinder the motion of the fluorophore and as a result an increased anisotropy value can be measured.

The steady-state anisotropy measurements could not distinguish between these two possible effects. It was not possible to see any evidence that the anisotropy of the hetero-dimer and hetero-trimer form of toxofilin–actin complex is significantly different which may indicate that the local effect of the toxofilin binding on the anisotropy was probably more pronounced.

The presence of two binding sites on toxofilin has to be taken into account in the interpretation of these affinity measurements (Fig. 1B). When we assume two independent and identical binding sites on toxofilin for actin the concentration of the binding sites are the double of the toxofilin concentration. The anisotropy values were plotted against the total concentration of binding site on toxofilin. By fitting Eq. (1) to the data points the calculated $K_D$ was 4.1 ± 1.7 μM.

The calculated $K_D$ value (4.1 ± 1.7 μM) is slightly higher than the $K_D$ value reported previously in the literature (2.3 ± 1.0 μM) [5]. The discrepancy between these values can be explained by considering that Delorme and colleagues assumed that there is only one binding site for actin on toxofilin. Repeating the analysis with the assumption of two identical binding sites on the toxofilin their $K_D$ value would be greater and would likely match the value reported in the present work.

The concentration dependence of the steady-state anisotropy (Fig. 1B) fitted well to a simple binding curve. The results indicated that the affinity of the two binding sites of toxofilin for actin are very similar. We could not exclude the possibility that there is a little difference between the two affinities that was not resolved in these experiments due to limitations posed by the nature of the applied method and the quality of data. We concluded therefore that toxofilin has two actin binding sites, and the affinities of these actin binding sites are identical or very close to each other.
decreased from 0.012 s$^{-1}$ (Fig. 2A). As the toxofilin concentration increased the release rate followed first order kinetic and was fitted with single exponentials $e^{-kt}$. The results of the fluorescence lifetime measurements showed that the actin-bound $\varepsilon$-ATP cannot be quenched dynamically in the presence of the toxofilin [13]. After fitting the data points with Eq. (2) the $K_{SV}$ value was found to decrease from 0.24 M$^{-1}$ to 0.03 M$^{-1}$ in the presence of toxofilin (Fig. 3). This result clearly indicates that the accessibility of the actin-bound $\varepsilon$-ATP is extensively reduced after the complex formation between the actin and toxofilin. These data may also suggest that parallel to these changes the nucleotide binding cleft on actin form a more compact conformation due to the binding of toxofilin.

Fig. 2. Effect of toxofilin on the release of bound $\varepsilon$-ATP from G-actin. (A) The fluorescence intensity decreased in time due to the release of the $\varepsilon$-ATP from G-actin. Single exponentials were fitted to see the rate constant for the $\varepsilon$-ATP release. The samples contained 1 $\mu$M G-actin (empty squares, $k_{obs_1} = 0.012$ s$^{-1}$) or 1 $\mu$M G-actin was supplemented with 6 $\mu$M toxofilin (grey circles, $k_{obs_2} = 0.0025$ s$^{-1}$). (B) The $k_{obs}$ value decreased from 0.012 s$^{-1}$ to 0.002 s$^{-1}$ as the toxofilin concentration was increased to 8 $\mu$M in 8 steps.

3.2. The effect of toxofilin on the nucleotide exchange on actin

The nucleotide exchange experiments were completed with $\varepsilon$-ATP actin monomers (1 $\mu$M) in the presence of different toxofilin concentrations (0–8 $\mu$M). $\varepsilon$-ATP was displaced from actin by ATP (1 mM) and the decrease of fluorescence intensity was followed after the release of the $\varepsilon$-ATP (Fig. 2A). The change in fluorescence followed first order kinetic and was fitted with single exponentials (Fig. 2A). As the toxofilin concentration increased the release rate decreased from 0.012 s$^{-1}$ to 0.0025 s$^{-1}$ (Fig. 2B) indicating that the binding of toxofilin slowed down the dissociation of $\varepsilon$-ATP from actin. Based on the determined $K_D$ value (4.1 $\mu$M) at least 72% of the actin was in complex with the toxofilin at the highest (8 $\mu$M) toxofilin concentration.

The results of the nucleotide exchange experiments showed that the binding of toxofilin to actin has functional consequences. One explanation for this can come from structural data about the toxofilin–G-actin complex. It was suggested that a segment of toxofilin (H3 helix) span across the nucleotide containing gap between the two major actin domains [6]. This structural brace over the nucleotide binding cleft may affect the in and out movement of the nucleotide adversely.

3.3. The effect of toxofilin on the accessibility of the bound nucleotide in actin

Steady-state fluorescence quenching experiments were performed to reveal the structural details behind the inhibition of the nucleotide exchange in the presence of toxofilin. This technique was applied previously to describe the structural consequences of cofilin or profilin binding to monomeric actin [13]. 5 $\mu$M $\varepsilon$-ATP labelled actin was quenched with acrylamide (added in 19 steps up to 0.3 M) in the absence and presence of 10 $\mu$M toxofilin. Under these conditions at least 73% of the actin was in complex with toxofilin. The results of the fluorescence lifetime measurements showed that the actin-bound $\varepsilon$-ATP cannot be quenched dynamically in the presence of the toxofilin [13]. Thermal stability of G-actin is correlated with the structure of the nucleotide binding cleft [18]. Differential scanning calorimetry measurements were completed to see the consequences of the toxofilin binding on the thermodynamic stability of G-actin. When 30 $\mu$M G-actin was present in the solution only one denaturation peak was identified at 57.2 °C (Fig. 4) which is similar to the data obtained before [19]. When 17 $\mu$M toxofilin was added to 30 $\mu$M actin (toxofilin bound actin is at least 47%) a new peak at 63.1 °C has appeared (Fig. 4). The new peak is clearly distinct from the $T_m$ of the un-complexed actin ($T_m = 57.2$ °C) and the incidental F-actin peak ($T_m = 67.4$ °C) and free toxofilin ($T_m = 53.1$ °C, curve not shown) as well [19].

The data suggest that the new peak (63.1 °C) belongs to the actin-toxofilin complexes. The higher $T_m$ value observed for the toxofilin bound actin monomers shows that the conformational change of the nucleotide binding cleft induced by the toxofilin is

Fig. 3. The Stern–Volmer plots of the steady-state quenching measurements with $\varepsilon$-ATP labelled actin monomers. The Stern–Volmer plot of $\varepsilon$-ATP labelled monomeric actin (5 $\mu$M) with acrylamide (0–0.3 M) in the absence of toxofilin (Fig. 3). Solid lines represent the fits obtained with Eq. (2). The calculated $K_{SV_S}$ value is 0.24 ± 0.05 M$^{-1}$ for the free actin bound $\varepsilon$-ATP molecules, and the $K_{SV_F}$ value is 0.03 ± 0.05 M$^{-1}$ in the presence of toxofilin.
accompanied by the increased thermodynamic stability of the actin monomers.

The DSC measurements were completed at toxofilin/actin molar ratio of about 1:2 where the peak of toxofilin free G-actin (57.2 °C) is absent on the denaturation curve obtained for G-actin in the presence of toxofilin. This result can further support the previous assumption that toxofilinPeptide contains at least two actin-binding sites and it can bind two actin monomers simultaneously.

4. Conclusions

Toxoplasma gondii is an intracellular parasite, which can affect the actin cytoskeleton of the invaded cells to promote its own mobility. This parasite produces special actin-binding proteins – such as toxofilin – to induce specific changes in the structure and dynamics of the actin cytoskeleton within the host cell. In the present work the effects of toxofilin on the centrally located nucleotide binding region of non-parasitic actin G-actin was characterised with spectroscopy techniques. Fluorescence anisotropy measurements proved that G-actin and toxofilin interact with 2:1 stoichiometry. Steady-state fluorescence anisotropy could reveal that the affinity of actin binding to the actin binding sites on toxofilin is 4.1 ± 1.7 μM. Kinetic assays showed that while toxofilin bound to non-parasitic actin monomers it was able to slow down the dissociation of the actin bound ATP. The fluorescence quenching experiments with actin bound ε-ATP indicated that toxofilin decreased the accessibility of the bound ε-ATP indicating that the nucleotide binding cleft is shifted to a closed conformation. The decreased nucleotide exchange rate in the presence of toxofilin can also be supported by the decreased accessibility of the nucleotide binding region of actin. The altered accessibility of the nucleotide binding pocket is accompanied by the increased thermal stability of the actin monomers suggesting that the effect of toxofilin expressed globally on the actin monomer. These observation provided a basis for the model in which the T. gondii collect lots of the host cell’s actin monomers at the beginning of the invasion and shift them into a stable, compact and functionally less effective conformational state. The decreased amount of polymerisation competent actin monomers after the invasion may decrease the responsiveness of the invaded cells. These results revealed important molecular changes underlying the patho-physiological consequences of T. gondii infection and showed how some parasites can complete their biological strategy after the invasion of an infected cell.

Acknowledgements

This study was supported by Grants from the Hungarian Science Foundation (OTKA) NN107776 and K112794 (to M.Ny.), Grants from the Hungarian National Office for Research and Technology (GVOP-3.2.1.-2004-04-0190/3.0 and GVOP-3.2.1.-2004-04-0228/3.0 (to M.Ny.)) and by the Grant of PTE AÖK-KA-2013/1 (to G.H.). This work was also supported by ‘Science, Please! Research Team on Innovation’ (SROP-4.2.2/08/1/2008-0011) program and by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11-1-2012-001 ‘National Excellence Programme’.

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