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

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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 ε-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.

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 (K_D = 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

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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$_{69-196}$ 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, $CaCl_2$, TRIZMA Base (tris-(hydroxy-methyl) aminomethane), $MgCl_2$, acrylamide, MOPS (3-(N-morpholino) propanesulfonic acid), imidazole, DOWEX 1x2-400, 2X YT Microbial Medium EZMix™ Powder, kanamycin, IPTG (isopropyl-β-D-thiogalactopyranoside) and thrombin were purchased from Sigma–Aldrich (Budapest, Hungary). ATP (adenosine-5′-tripho sphate), MEA (mercaptoethanol), and NaN_3 (sodium azide) were supplied by Merck (Budapest, Hungary). The ε-ATP ($1,N^6$-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^{2+}-G-actin was stored in buffer A (4 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA, 0.005% NaN_3, pH 8.0). The concentration of the G-actin was determined by using the absorption coefficient of 1.11 mg ml^{-1} cm^{-1} at 280 nm [11]. All the experiments were completed in the presence of Ca^{2+}-G-actin.

The DNA fragment encoding toxofilin$_{69-196}$ 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™ 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 was 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$_{69-196}$ (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^{-1} cm^{-1} (ProtParam).

The labelling of actin with ε-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]:

$$\frac{s_{actual}-s_{min}}{s_{max}-s_{min}}=\frac{([L_{total}]+[P_{total}]+K_D)-\sqrt{([L_{total}]+[P_{total}]+K_D)^2-4[P_{total}][L_{total}]}}{2[P_{total}]} \quad (1)$$

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

In case of the fluorescence anisotropy measurement the s_{actual} is the measured anisotropy value (r) at a certain toxofilin concentration, s_{min} represents the anisotropy of the un-complexed actin monomer (r_{min}), and s_{max} is related to the highest anisotropy value (r_{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:

$$\frac{F_0}{F}=\left(\sum_{i=1}^{n}\frac{f_i}{(1+K_{SV_{Si}}[Q])(1+K_{SV_{Di}}[Q])}\right)^{-1} \quad (2)$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity at different quencher concentration (Q) and $K_{SV_{Si}}$ and $K_{SV_{Di}}$ are the static and dynamic Stern–Volmer constant of the i^{th} population represented by the fraction of f_i [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 ε-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 ε-ATP and toxofilin with a final concentration between 0 and 8 μM. To displace the bound ε-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$_{(69-196)}$) of toxofilin was used that can be related