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Solution structure and dynamics of ADF from *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii ADF (TgADF) belongs to a functional subtype characterized by strong G-actin sequestering activity and low F-actin severing activity. Among the characterized ADF/cofilin proteins, TgADF has the shortest length and is missing a C-terminal helix implicated in F-actin binding. In order to understand its characteristic properties, we have determined the solution structure of TgADF and studied its backbone dynamics from ^{15}N-relaxation measurements. TgADF has conserved ADF/cofilin fold consisting of a central mixed β-sheet comprised of six β-strands that are partially surrounded by three α-helices and a C-terminal helical turn. The high G-actin sequestering activity of TgADF relies on highly structurally and dynamically optimized interactions between G-actin and G-actin binding surface of TgADF. The equilibrium dissociation constant for TgADF and rabbit muscle G-actin was 23.81 nM, as measured by ITC, which reflects very strong affinity of TgADF and G-actin interactions. The F-actin binding site of TgADF is partially formed, with a shortened F-loop that does not project out of the ellipsoid structure and a C-terminal helical turn in place of the C-terminal helix α4. Yet, it is more rigid than the F-actin binding site of *Leishmania donovani* cofilin. Experimental observations and structural features do not support the interaction of PIP2 with TgADF, and PIP2 does not affect the interaction of TgADF with G-actin. Overall, this study suggests that conformational flexibility of G-actin binding sites enhances the affinity of TgADF for G-actin, while conformational rigidity of F-actin binding sites of conventional ADF/cofilins is necessary for stable binding to F-actin.

1. Introduction

The proteins of the ADF/cofilin family are one of the key regulators of actin filament dynamics and have been shown to be essential for several eukaryotes (Ono, 2007). ADF/cofilin proteins increase the turnover rate of actin filaments by accelerating the dissociation of actin monomers from the filament pointed end. In addition, F-actin filaments are actively severed by several members of the ADF/cofilin family proteins, leading to the generation of new uncapped barbed ends that immediately start growing. The ADF/cofilin proteins manifest their activity through G-actin binding (Lappalainen et al., 1997), F-actin binding and depolymerization (Lappalainen et al., 1997), F-actin severing (Andrianantoandro and Pollard, 2006), controlling the rate of nucleotide exchange from actin monomer (Andrianantoandro and Pollard, 2006, Hawkins et al., 1993; Hayden, et al. 1993; Nishida, 1985; Yamashiro et al., 2005), and actin monomer sequestering activity (Chen et al., 2004; Mehta and Sibley, 2010; Nachmias, 1993; Yamashiro et al., 2005). ADF/cofilin proteins show pH sensitivity that is more pronounced in case of the vertebrates than other eukaryotes. The F-actin depolymerizing activity (Yonezawa et al., 1985) of ADF/cofilin proteins is higher at pH ~8 compared to pH ~6.8 (Pope et al., 2004). However, the activity of ADF/cofilin from *Leishmania donovani* and *Toxoplasma gondii* are pH independent (Mehta and Sibley, 2010; Tammana et al., 2008).

The non-vertebrate ADF/cofilins represent the highly conserved ADF-homology (ADF-H) fold, which is also observed in destrin, actophorin, and gelsolin. The structure of 143 residues *Saccharomyces cerevisiae* cofilin consists of a six stranded mixed β-sheet, in which the four central strands are anti-parallel while the two edge strands run parallel to the neighboring strands. The β-sheet is sandwiched between a pair of α-helices on each face. The longest helix (α3) is kinked at the position of a serine residue in a conserved segment. ADF/cofilins have two distinct actin-binding sites. These have been named as the G/F-site and the F-site (Ono, 2003). The G/F-site of ADF/cofilins is required for binding to both the G-actin and the F-actin. This binding site corresponds to helix α3 (long kinked helix), the N-terminal flexible region, the strand β5, and the loop before the C-terminal helix (α4) (Paavilainen et al.,

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2008). The F-site is responsible for binding to F-actin and F-actin severing activity. In conventional ADF/cofilins, the F-site is comprised of the β3–β4 loop, also called the 'F-loop', C-terminal helix (helix α4), and the C-terminus. The F-loop typically projects out of the structure and is predicted to intercalate within the actin filament. Sites of ADF/cofilin involved in actin binding have been confirmed by several mutational analysis (Moriyama and Yahara, 1999; Moriyama et al., 1992), cross-linking (Yonezawa et al., 1991a,b), and peptide competition or synchrotron electron footprinting (Guan et al., 2002). The G/F-actin binding sites and the F-actin binding sites cluster together in the three-dimensional structure (Fedorov et al., 1997; Hatanaka et al., 1996; Pope et al., 2000) to constitute respective actin binding surfaces.

Vertebrate cofilins are ~160 residues long and in comparison to non-vertebrate subgroup, these have two sequence inserts and an extension of C-terminus. In chick cofilin, human cofilin, and destrin, the first insert is an additional segment bearing helix (α2), in between α1 and β2, which contains a putative nuclear localization sequence (NLS). A second seven residue insert follows β3. In chick cofilin, this insert forms a bulge followed by an additional β-strand that pairs with the N-terminal end of β2 and expands the central β sheet scaffold (Gorbatyuk et al., 2006). Vertebrate ADF/cofilins also contain a C-terminal extension of approximately eight residues. In chick cofilin, this extension forms a β-hairpin comprising antiparallel strands β7 and β8 that nestle between β4 and α6 (Gorbatyuk et al., 2006). The sequence of the C-terminal extension may be unique to vertebrate cofilins and it may impart unique F-actin binding properties.

Various activities of ADF/cofilin family proteins have been shown to be dependent on the affinity of these proteins for the actin filaments. It has been observed in the case of the human cofilin that a point mutation of a basic residue K96 in the F-loop leads to a loss of severing activity and increased depolymerizing activity (Pope et al., 2000). Similarly it has been reported that by mutation, in the F-actin binding sites of ADF/cofilin proteins, severing and depolymerizing activities can be uncoupled (Ono et al., 1999, 2001; Pope et al., 2000). In the case of *Schizosaccharomyces pombe* cofilin, it has been found that the point mutation of R78 in the F-loop resulted in a loss of nucleating activity (Andrianantoandro and Pollard, 2006).

Intracellular protozoan parasites that belong to the phylum Apicomplexa are a significant cause of disease in humans and animals (Joynson and Wreghitt, 2001). Apicomplexa parasites utilize a unique mode of motility, termed gliding, to move across epithelial barriers and translocate into the host cytoplasm (Barragan and Sibley, 2003). Gliding motility is conserved across the Apicomplexa, is dependent on the turnover of actin filaments, and is inhibited by treatment of the parasites with cytochalasin D (Dobrowolski and Sibley, 1996). Although, Apicomplexa actins characterized so far exhibit high sequence homology to the vertebrate actins, they are in contrast typically inherently unstable (Sahoo et al., 2006; Schmitz et al., 2005; Schuler et al., 2005), have significantly lower critical concentration for polymerization (Sahoo et al., 2006), and yet are almost exclusively unpolymerized in the cytoplasm (Dobrowolski et al., 1997; Sahoo et al., 2006; Wetzel et al., 2003). For example, 98% of actin is present as G-actin in *T. gondii* (Dobrowolski et al., 1997; Sahoo et al., 2006; Wetzel et al., 2003), in comparison to higher eukaryotes where up to 50% cellular actin is in the filament form. Despite the presence of G-actin in high cellular concentration, formation and turnover of filamentous actin is essential for both gliding motility and the host cell invasion by *T. gondii* (Poupel and Tardieux, 1999; Shaw and Tilney, 1999; Wetzel et al., 2003).

T. gondii expresses a conserved homolog of the ADF/cofilin family (TgADF) that has been shown to play a critical role in actin monomer sequestering and filament disassembly (Mehta and Sibley, 2010). TgADF does not co-sediment with actin filaments. However, it does disassemble actin filaments in pH independent manner but does not form a stable association with actin filaments. TgADF possesses low severing activity, but it inhibits nucleation and polymerization, primarily due to sequestering of actin monomers.

The genomes of Apicomplexa *T. gondii*, *Eimeria tenella*, and *Neospora caninum* express ADF/cofilin proteins that are only 118 residues in length. In these ADF/cofilin proteins, the G/F-actin binding sites are significantly conserved, but there are significant deletions of residues that are required to form the sites for binding F-actin. Specifically, these apicomplexan ADF/cofilins possess a truncated C-terminus and lack the C-terminal charged residues that are predicted as necessary to bind to F-actin. The F-loop is itself much shorter and lacks the conserved basic residue for interaction with F-actin. *Plasmodium* contains two members of the ADF/cofilin family. In *Plasmodium falciparum*, the larger of the two, termed as PfADF2, is a 143 residue protein which is closer to the conventional yeast type ADF/cofilin (Schüler et al., 2005). The shorter ADF, termed as PfADF1, consists of 122 residues, and closely resembles ADF from the other apicomplexans described above. The shorter apicomplexan TgADF protein is among the smallest of the ADF/cofilin family and has not been structurally characterized so far.

In our present study, we have determined the solution structure of TgADF using NMR spectroscopy. Further, we have measured backbone ^{15}N-relaxation rates and have analyzed the dynamics of TgADF in solution using the Lipari–Szabo formalism. We have also determined the thermodynamic parameters characterizing the interaction of TgADF with rabbit muscle ADP–G-actin using isothermal titration calorimetry (ITC). We also examined the effect of dioctanoyl phosphatidylinositol-4,5-bisphosphate (PIP2), on TgADF itself, and on binding of TgADF to rabbit muscle ADP–G-actin, using ITC and NMR experiments. In order to better understand the structure–function relationship, we have docked the solution structure of TgADF on the crystal structure of G-actin monomer. The docking study has provided us with important insights into the dynamic regulation of structural interaction that lead to high affinity interactions between TgADF and G-actin interactions.

2. Materials and methods

2.1. Preparation of NMR samples

The TgADF protein is composed of 118 amino acid residues with an additional N-terminal twenty-one residue purification tag making the molecular weight of the tagged protein equal to 15,480 Da. TgADF was cloned by Mehta and Sibley (2010). The clone was over-expressed in BL21 (λDE3) strain of *Escherichia coli*. Conditions for optimal over-expression and purification were standardized. The yield of purified protein was 30 mg/L of culture medium. For isotopic labeling, over-expressed TgADF was standardized in minimal media containing ^{15}N-ammonium sulfate and ^{13}C-glucose (CIL, MA, USA) as the sole nitrogen and carbon sources, respectively. The additional twenty-one residue purification tag was cleaved by Factor Xa protease at site IEGR according to the manufacturer's guidelines.

NMR samples of ^{13}C/^{15}N-labeled TgADF were prepared at concentration of approximately 0.8 mM in NMR buffer (20 mM sodium phosphate pH 6.5, 50 mM NaCl, 1 mM DTT, 0.1% NaN_3, and 1 mM AEBSF) containing 95% H_2O/5% ^2H_2O.

Dioctanoyl-PI(4,5)P2 sample was prepared in G-actin buffer (2.0 mM HEPES, 0.2 mM $CaCl_2$, 0.2 mM ADP, 1.0 mM NaN_3, 1.0 mM BME, pH 7.4) at a concentration of 1 mg/mL (1.34 mM).