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Assembly of *Bacillus subtilis* FtsA: Effects of pH, ionic strength and nucleotides on FtsA assembly

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ABSTRACT

In this work, the assembly of purified *Bacillus subtilis* FtsA was analyzed by several complimentary techniques. FtsA assembled to form filaments and bundles and the polymers disassembled upon dilution. FtsA assembled more efficiently at pH 6.0 as compared to that at pH 7.0 or 8.0 and high salt inhibited the assembly of FtsA. FtsA was found to hydrolyze ATP *in vitro*; however, neither ATP nor ADP influenced the assembly kinetics of FtsA. Though FtsA is a homologue of actin, cytochalasin D did not inhibit the assembly of FtsA. Interestingly, a hydrophobic molecule, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, inhibited the assembly of FtsA.

1. Introduction

Bacterial cell division is a well-coordinated process where a parent cell divides giving rise to two daughter cells. The first stage in the cell division is the formation of a Z-ring at the mid cell by FtsZ. The formation and dynamics of the Z-ring are precisely regulated by the action of positive and the negative regulators of FtsZ assembly [1–7]. FtsA, SepF, ZipA and ZapA promote Z-ring assembly and hence are referred to as the positive regulators of the Z-ring assembly [1,2,6,7]. EzrA, MinC, MinD and nucleoid occlusion on the other hand inhibit the Z-ring formation and are referred to as the negative regulators of Z-ring assembly [3,4,8].

FtsA, one of the positive regulators of FtsZ assembly, is present in both Gram-positive and Gram-negative bacteria. FtsA has also been shown to interact with FtsZ [9] and colocalize with FtsZ at the mid cell [10,11]. FtsA is thought to play a key role in the assembly and stabilization of the Z-ring during bacterial cytokinesis [12].

FtsA belongs to the actin/Hsc70/sugar kinase ATPase superfamily and is structurally homologous to actin [13]. It has the conserved ATP binding motif VLTGG, and FtsA from *Escherichia coli*, *Bacillus subtilis* and *Thermotoga maritima* have been shown to bind ATP *in vitro* [11,14,15]. However, the ATP hydrolysis activity of FtsA has been confirmed only for *Bacillus subtilis* and *Pseudomonas aeruginosa* proteins [11,16]. Since FtsA shows similarities to actin and belongs to the actin family of proteins, it was always questioned if FtsA could polymerize like actin. In the past years various laboratories have reported FtsA polymerization and have also suggested the role of FtsA assembly in bacterial cytokinesis [17–19]. Shiomi et al. showed that while dimeric FtsA stabilized the Z-ring; monomeric FtsA had an inhibitory effect on the Z-ring assembly [20]. In an earlier study a mutant of *E. coli* FtsA (R286W; FtsA*), having an increased self-interaction ability, showed an enhanced effect on the stability of the Z-ring in the cells. The mutant FtsA* was able to bypass the requirement for ZipA or FtsK in the Z-ring formation [21]. Interestingly, *Ec*FtsA* inhibited the assembly of FtsZ and depolymerized the preformed FtsZ polymers in the presence of ATP *in vitro* [22] indicating that ATP may be playing a role in the interaction of FtsA with FtsZ or other cell division proteins during cell division.

The C-terminal of FtsA is highly conserved among different species of bacteria and has been reported to be responsible for the localization of FtsA on the membrane [23,24]. Further, it was also found that the C-terminal truncated FtsA formed rod like structures in the cytoplasm, validating the self-interaction ability of FtsA [23,24]. Pichoff et al. have recently reported that FtsA may have a role in recruiting the downstream proteins in cell division and the self-interaction of FtsA may hamper its ability to do so [25]. Using electron microscopy, it has been shown that FtsA forms filaments inside the cells. It is proposed that these FtsA filaments may form an "A-ring" which attaches Z-ring to the membrane [18]. FtsA mutants (K145A, M147E and I278K) that are unable to self interact have been found to be deficient in polymerization [18]. These

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mutants caused cell elongation and a reduction in the fraction of successful cell divisions as compared to the wild type [18]. Additionally, these polymerization deficient mutants were also unable to complement a ts-FtsA mutant at non-permissive temperature suggesting that FtsA polymerization is essential for its biological function [18] and FtsA polymers play an important role in bacterial cytokinesis.

In this work, we studied FtsA assembly and characterized FtsA polymers. We found that FtsA hydrolyzes ATP and that ATP had no effect on the assembly kinetics of FtsA. Unlike FtsZ protofilaments or microtubules, FtsA polymers were found to be cold stable; however, they disassembled upon dilution similar to the FtsZ protofilaments and microtubules. Though FtsA shares structural similarities with eukaryotic protein actin and has been recently reported to form actin like filaments [18], we found that cytochalasin D had no effect on the assembly of FtsA suggesting that FtsA may have different assembly dynamics than actin. Further, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS), a hydrophobic compound, was found to inhibit FtsA assembly suggesting that the polymerization of FtsA may predominantly involve hydrophobic interactions. We also found that the assembly of FtsA was pH dependent with FtsA showing higher assembly at lower pH.

2. Materials and methods

2.1. Materials

Piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes), phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), β-mercaptoethanol (β-ME), guanosine 5′-triphosphate (GTP) and adenosine 5′-triphosphate (ATP) were purchased from Sigma. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Calbiochem. 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) was purchased from Molecular Probes. Ni-NTA coupled to Sepharose® was purchased from Qiagen. Bio-Gel P6 was obtained from Bio-Rad. All other chemicals used were of analytical grade. *Pfu*turbo DNA polymerase was obtained from Stratagene. *E. coli* BL21(DE3) [B F^- *dcm ompT hsdS* (rB^-, mB) *gal* λ(DE3)] and plasmid *p*ET16b(+) were from Novagen.

2.2. Cloning and overexpression of FtsA

The genomic DNA of *Bacillus subtilis* ATCC 6633 was used for PCR amplification of *fts*A using *Pfu*turbo DNA polymerase. The primers 5′-AGGTGCCATCATATGAACAACAATGAACTTTAC-3′ and 5′-ATGAGGATCCATTTATTCCCAAAACATGC-3′ were used to amplify the coding sequence of *fts*A. The PCR product was cloned into *Nde*I and *BamH*I site of *p*ET16b(+) vector (*p*ET16b-*fts*A). *Escherichia coli* (*E. coli*) DH5α cells were used as the cloning host. *E. coli* BL21(DE3) *p*LysS cells were transformed with the recombinant plasmid for overexpression and purification of FtsA. The nucleotide sequences of the genomic *fts*A from the *B. subtilis* ATCC 6633 strain used for cloning and from selected *p*ET16b-*fts*A clones were confirmed to be identical, using an automated DNA sequencer (ABI-PRISM 377). The nucleotide sequence of the cloned *ftsA* also matched exactly with the genomic *fts*A (Gene ID 936145) sequence of *B. subtilis* 168 (GenBank accession number AL009126.3).

2.3. Isolation and purification of FtsA

Recombinant FtsA protein from *Bacillus subtilis* was overexpressed and purified from *E. coli* BL21(DE3) *p*LysS cells. The cells containing the desired gene were grown at 37 °C in LB media containing 100 μg mL^{-1} of ampicillin and 12.5 μg mL^{-1} of chloramphenicol. The cells were induced at late log phase ($O.D_{600}$ ~0.8) using 1 mM IPTG and incubated for an additional 6 h. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The cell pellet was washed and resuspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole, pH 8.0) containing lysozyme 0.25 mg mL^{-1}, 0.1% β-ME, 1 mM PMSF and 10% glycerol and homogenized on ice. The cells were disrupted by sonication with 30 pulses at 30 s interval 10 times. The cell lysate was cleared by centrifugation at 120,000 × *g* at 4 °C for 20 min. The cleared suspension was loaded on a Ni-NTA agarose affinity column pre equilibrated with lysis buffer and incubated for 30 min at 4 °C. The column was washed extensively with 4 column volume of buffer C (50 mM NaH_2PO_4, 500 mM NaCl, 10% glycerol, 50 mM imidazole pH 8.0) followed by 3 column volume of buffer D (100 mM NaH_2PO_4, 500 mM NaCl 10% glycerol, 100 mM imidazole, pH 8.0). The bound protein was then eluted with buffer E (100 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was desalted using Biogel P-6 resin equilibrated with 25 mM Tris, 100 mM KCl pH 8.0 and then concentrated using Amicon® Ultra PL-10 (Millipore) at 4 °C. A single band on the coomassie blue stained SDS–PAGE suggested that the protein was pure (Fig. 1a). Additionally, the eluted protein was analyzed by western blot using an anti-polyhistidine antibody (Fig. 1b). The concentration of the purified protein was determined by Bradford method using BSA as standard [26]. FtsA was aliquot and stored at −80 °C.

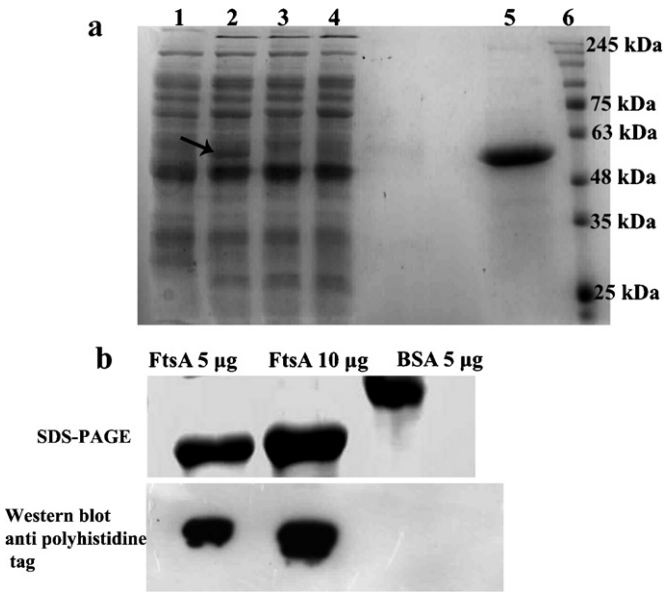

Fig. 1. Expression and purification of FtsA. (a) FtsA was purified using Ni-NTA agarose column. The different steps in FtsA purification were analyzed on 10% SDS–PAGE. The lanes 1, 2, 3, 4, 5 and 6 represent cleared cell lysate of uninduced culture, cleared cell lysate of induced culture, flowthrough of cell lysate from Ni-NTA column, wash with buffer containing 50 mM imidazole, FtsA eluted with 250 mM imidazole and molecular weight markers, respectively. The arrow represents the overexpressed FtsA in the induced culture. (b) Purified FtsA was also analyzed by SDS–PAGE and Western blot. Purified his-tagged FtsA (5 and 10 μg) was run on a 10% SDS–PAGE and analyzed by coomassie staining and Western blot using anti polyhistidine antibody.

2.4. Assay for ATPase activity of FtsA

ATPase activity of FtsA was monitored using malachite green ammonium molybdate assay [27,28]. Briefly, FtsA (8 μM) was incubated with 1 mM ATP in the presence of 25 mM Tris–HCl pH 7.2, 100 mM KCl, and 2 mM $MgCl_2$ at 37 °C. After different time intervals (5, 10, 15, 20, 25 and 30 min), a 40 μL aliquot of each of the