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Research paper

Dimethyl sulphoxide and Ca^{2+} stimulate assembly of *Vibrio cholerae* FtsZ

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ABSTRACT

We cloned, overexpressed and purified *Vibrio cholerae* FtsZ protein for the first time. We used several complementary techniques to probe and compare the comparative assembly properties of recombinant *V. cholerae* FtsZ (VcFtsZ) and *Escherichia coli* FtsZ (EcFtsZ). We observed that VcFtsZ polymerized at a slower rate than EcFtsZ and interestingly its polymerization was highly dependent on the presence of Ca^{2+} ion. Furthermore, DMSO specifically modulated the polymerization of VcFtsZ, promoted polymer bundling and increased the stability of the VcFtsZ protofilaments. Whereas DMSO showed no significant stimulatory effect on the assembly and bundling of EcFtsZ. Transmission electron microscopy experiments demonstrated that in presence of 8% DMSO the average thickness of the VcFtsZ polymers were increased significantly. DMSO specifically stabilized the VcFtsZ polymers against dilution induced disassembly and it reduced the GTPase activity of VcFtsZ. These results collectively suggested that despite lot of sequence homology, the assembly of VcFtsZ and EcFtsZ are differently regulated processes. We expect to use this knowledge of assembly properties of VcFtsZ for screening of small molecules against VcFtsZ for development of anti-cholera agent.

1. Introduction

Cholera an acute watery diarrheal disease, caused by *Vibrio cholerae*, is a major global public health problem in developing countries, causing an estimated 120,000 deaths per year [1–3], *WHO annual report 1995*, *2004*, *2008*. Though significant progress has been achieved in the management of diarrheal diseases by improved hygiene, development of new antimicrobials and vaccines, the death burden remains the same especially in children below 5 years of age [*WHO Annual Report*, *1995*]. Moreover, emergence of multiple drugs resistant *V. cholerae* strains has made many of the available anti-cholera drugs ineffective [4]. Accordingly, it has now become important to identify and characterize novel and specific drug targets which could be used to selectively kill cholera pathogens. In recent years, attempts have been made to target bacterial cell division machinery by targeting one of the major bacterial cell division proteins FtsZ which assembles in a finely regulated manner at the cell division site.

Bacterial cytokinesis has long been used as a target for drug development [5–11]. Cytokinesis in bacteria is effected by a complex macromolecular machinery, called divisome, containing seven or more conserved proteins which assemble with a definite dependence hierarchy at the site of cell division [9,12]. Divisome assembly is initiated by the polymerization of tubulin homologue FtsZ into a ring like structure, called 'Z-ring' which serves as a scaffold for the recruitment of the downstream components of the divisome and persists throughout cell division guiding the synthesis, location and shape of the division septum [12]. FtsZ, a 40-kDa protein (in *Escherichia coli)* is ubiquitous in all free-living eubacteria and archaea [13,14]. Its wide distribution among the prokaryotes and high sequence homology suggest that it probably plays similar conserved role in prokaryotes [15–17]. The indispensability of FtsZ in prokaryotic cell division has long been established by several genetic studies [9,18,19].

V. cholerae is a Gram-negative facultative anaerobic rod shaped microorganism, which divides faster than many commonly known bacteria like *E. coli*. While the normal generation time for *E. coli* is approximately 30 min, under optimal condition *V. cholerae* requires 18 min to multiply [21]. So this fast growth rate of *V. cholerae* compared to the other bacteria may be attributed by its cell division

Abbreviations: PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; EGTA, ethylene bis-(oxyethylenenitrilo) tetraacetic acid; GTP, guanosine 5′-triphosphate; PMSF, phenylmethylsulfonyl fluoride; Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetic acid; BSA, bovine serum albumin.

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machinery which predominantly comprises of FtsZ. Although in previous years attempts have been made to understand the assembly properties of *E. coli* FtsZ (EcFtsZ), there exists no such reports which addressed the *V. cholerae* FtsZ assembly properties. Moreover despite higher sequence homology, different FtsZs from different microorganisms differ considerably in their assembly properties. For example, divalent calcium was found to promote EcFtsZ assembly strongly while it has no such effect on MtbFtsZ polymerization [22,23]. For screening of compounds against *V. cholerae* FtsZ (VcFtsZ) for the development of anti-bacterial agents, we need to understand the assembly properties of VcFtsZ. So, to probe the assembly properties of *V. cholerae* FtsZ (VcFtsZ) and to see how does it differ from its *E. coli* homolog, we first time cloned, overexpressed and purified the VcFtsZ protein from *E. coli BL21(DE3)/pLysS* cells. We used several complementary techniques to probe the assembly properties of recombinant *V. cholerae* FtsZ (VcFtsZ) and *E. coli* FtsZ (EcFtsZ). Interestingly, polymerization of VcFtsZ *in vitro* was found to be highly dependent on the presence of Ca^{2+} *in vitro*. Furthermore, DMSO exerted a strikingly different effect on the assembly of VcFtsZ as compared to EcFtsZ. It specifically modulated the polymerization of VcFtsZ, promoted bundling and increased the stability of the VcFtsZ protofilaments. Whereas, as previously reported we observed that DMSO showed no significant stimulatory effect on the assembly and bundling of EcFtsZ [24]. These results indicate that although VcFtsZ and EcFtsZ have 76% sequence homology but the assembly properties of VcFtsZ and EcFtsZ are differently regulated process.

2. Materials and methods

2.1. Materials

PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), isopropyl-β-D-thiogalactopyranoside (IPTG), spectroscopy grade dimethyl sulphoxide (DMSO) and guanosine 5′-triphosphate (GTP) were obtained from Sisco Research Laboratories. $MgCl_2$, $CaCl_2$, NaCl, Malachite green, sodium citrate and ammonium molybdate were purchased from Merck, Germany. Phenylmethylsulfonyl fluoride (PMSF), EGTA and monoclonal HRP conjugated Anti-polyHistidine antibody were purchased from Sigma, USA. Ni^{2+}-nitrilotriacetic acid (NTA) agarose column was purchased from Qiagen, USA. All other chemicals used were of analytical grade and were purchased from Sisco Research Laboratories, India.

2.2. Bacterial strains used

E. coli host strain BL21(DE3)/pLysS (Novagen, USA) was used for the regulated expression of both *V. cholerae* FtsZ and *E. coli* FtsZ. *E. coli* strains DH5α and XL1 blue were used for DNA propagation and cloning purpose.

2.3. Cloning, expression and purification of VcFtsZ

V. cholerae FtsZ gene was PCR amplified from *V. cholerae* O1 biovar El Tor str. N16961 genome using primers 5'-GCGCATATGTTTGAACCGATGATGG-3' and 5'-AAGAAGCTTTTAGTCAGCTTGACGTCTCA-3'. The ~1.2 kb fragment was double digested and ligated into the *Nde*I-*Hind*III sites of pET-24a vector (Novagen) to construct VcFtsZ expression plasmid pAC-Vz and sequence was verified. Recombinant *V. cholerae* FtsZ was over-expressed and purified from *E. coli* BL21(DE3)/pLysS cells. Briefly, BL21(DE3)/pLysS cells harboring pAC-Vz were grown at 37 °C in Luria Bertani (LB) broth containing 50 μg/ml kanamycin until they reached early log phase (A_{600} ~ 0.4–0.6) when protein expression was induced by the addition of 1 mM IPTG for an additional 3 h. Cells were harvested by centrifugation at 12,000× *g* at 4 °C and resuspended in lysis buffer (50 mM Tris (pH 8), 300 mM NaCl, 2 mM $MgSO_4$, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and a cocktail of protease inhibitor) on ice. Following 1 h of incubation in ice, cells were disrupted by sonication with 15-s pulses at 30-s intervals between the pulses for five to eight cycles. The insoluble debris were removed by centrifugation at 12,000× *g* at 4 °C for 30 min. After centrifugation, the clear supernatant was loaded onto a 5 mL Ni^{2+}-NTA agarose column (for 1 L culture), pre-equilibrated with lysis buffer. The column was washed with about 10 column volumes of wash buffer (50 mM Tris,pH 8, 300 mM NaCl, 40 mM imidazole) and the bound protein was eluted with a 100–400 mM imidazole gradient in 50 mM Tris (pH 8.0) buffer containing 300 mM NaCl. Fractions were collected and analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The peak fractions containing pure VcFtsZ-HT protein were pooled and dialyzed against 25 mM PIPES-NaOH buffer (pH 6.9) containing 100 mM KCl, 0.1 mM EDTA and 1 mM dithiothreitol (DTT) and 10% glycerol at 4 °C. The N-terminal six-His-tag was removed by digestion with 2 units of thrombin (GE Healthcare, USA)/ml of VcFtsZ on ice [25]. Thrombin was removed by passing the digested protein mixture through a benzamidine sepharose column (GE Healthcare, USA; flow rate, 1 mL/min) equilibrated with desalting buffer containing 25 mM PIPES-NaOH (pH 6.9), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol [25]. Thrombin digested VcFtsZ protein was further dialyzed against 25 mM PIPES-NaOH buffer (pH 6.9) at 4 °C. The VcFtsZ-HT protein obtained by this method was ~98% pure, as judged by SDS-PAGE. The concentration of VcFtsZ protein was determined by the Bradford's method [26] using BSA as the standard. The BSA concentration was also calibrated by ultraviolet (UV) spectroscopy method. This calibrated BSA standard was used to calibrate the Bradford's assay.

2.4. Purification of EcFtsZ

Recombinant wild type EcFtsZ protein was overexpressed and purified from *E. coli* BL21(DE3)/pLysS cells (a generous gift from Prof. HP Erickson, Duke University,USA) as described previously with some modifications [10,23,24]. Briefly, *E. coli* BL21(DE3)/pLysS cells containing EcFtsZ expression plasmid were grown in LB broth until they reach early log phase (A_{600} ~ 0.6) and then cells were induced with 0.5 mM IPTG for another 4 h. Cells were harvested by centrifugation and lysed by sonication with 10-s pulses at 30-s intervals between the pulses for five cycles following resuspension into lysis buffer (50 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and 1 mM $MgSO_4$ and a cocktail of protease inhibitor) on ice. The sonicated cell lysate was then centrifuged at 12,000× *g* at 4 °C for 30 min to remove the cell debries. The supernatant was subjected to 25% ammonium sulfate cut and incubated on ice for another 30 min and then ultra-centrifuged at 30,000× *g* at 4 °C for 30 min. The pellet was resuspended in 25 mM PIPES-NaOH buffer (pH 6.9) and dialyzed against the same buffer for 6 h. The dialyzed protein was further purified by one-cycle of temperature-dependent glutamate induced polymerization and depolymerization method as described previously [10,23,24]. The polymer pellet was resuspended in 25 mM PIPES buffer in ice. EcFtsZ protein obtained by this method was >95% pure, as judged by SDS-PAGE. The protein concentration was determined by the Bradford method [26] using BSA as the standard.

2.5. Western blot analysis

The VcFtsZ protein was identified and confirmed by Western Blot method, originally described by Minotti et al. [27] with some

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