In Vitro Reconstitution of the Initial Stages of the Bacterial Cell Division Machinery

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Abstract Fission of many prokaryotes as well as some eukaryotic organelles depends on the self-assembly of the FtsZ protein into a membrane-associated ring structure early in the division process. Different components of the machinery are then sequentially recruited. Although the assembly order has been established, the molecular interactions and the understanding of the force-generating mechanism of this dividing machinery have remained elusive. It is desirable to develop simple reconstituted systems that attempt to reproduce, at least partially, some of the stages of the process. High-resolution studies of *Escherichia coli* FtsZ filaments’ structure and dynamics on mica have allowed the identification of relevant interactions between filaments that suggest a mechanism by which the polymers could generate force on the membrane. Reconstituting the membrane-anchoring protein ZipA on *E. coli* lipid membrane on surfaces is now providing information on how the membrane
attachment regulates FtsZ polymer dynamics and indicates the important role played by the lipid composition of the membrane.

**Keywords** Atomic force microscope · FtsZ · Bacterial cytoskeleton · Theoretical modeling · Planar lipid bilayers

1 Introduction

Bacterial cell division is a highly orchestrated biological process through which a cell is divided into two daughter cells. The process occurs by the formation of a multiprotein complex at the midcell region of the bacteria [1]. This divisome is constituted by at least ten proteins that assemble in a hierarchical linear order [2]. The different constituents of the machinery have been identified and their recruiting order established, but a quantitative description of their molecular interactions is not yet available and the force-generating mechanism is still unknown. The small size of bacteria and organelles and the large number of components of the machinery make it challenging experimentally to approach the study in a more quantitative way. It is, therefore, desirable to have access to more simple reconstituted systems that partially reproduce some of the stages of the process. Biophysical surface characterization and single molecule techniques provide experimental data that can be modeled theoretically. This approach has been used in the study of eukaryotic cytoskeleton proteins and has provided a wealth of information related to the mechanical properties and molecular mechanisms underlying their rich biological functions, which go from a pure mechanical role, serving as a scaffold to retain cellular shape, to an active role as motors that contract, exert force, and displace different cellular organelles and structures in a highly regulated and directional manner [3–7]. The in-depth characterization and understanding of these biological systems opens the possibility of incorporating them into devices and exploiting their mechanochemical properties to transport and distribute cargo on structured surfaces with high spatial control [8, 9].

Within the last few years, it has become evident that bacteria also have a complex and rich cytoskeleton, understanding as cytoskeleton polymeric filamentous structures of a single class of protein that have a long-range order within the cell [10]. Although great progress has been made in determining the structure of the monomers and understanding their polymerization process, their dynamic behavior and mechanical properties are not yet as well-understood as those of their eukaryotic counterparts. Out of the several bacterial cytoskeletal proteins known to be capable of self-assembling in vitro into extended polymeric filaments, FtsZ is known to be essential for bacterial cell division [11–13]. This protein presents strong similarities in its crystal structure with eukaryotic tubulin and shares its GTPase activity required for polymerization [14], in spite of having only 10% sequence homology. It assembles into a dynamic ring-like, membrane-bound structure at the midcell region of the bacteria [15]. In vitro assays have provided information about the polymerization process, but there are still important unanswered questions regarding the dynamic behavior of the polymers, how they assemble and disassemble, the role played by GTP hydrolysis, and the mechanism of force generation [13–15].

We approach the study of FtsZ polymerization on surfaces using atomic force microscopy to characterize the structure and dynamic behavior of individual FtsZ filaments on different surfaces. First, the filaments were observed and characterized on a mica surface. In an effort to simulate the in vivo surface environment, the membrane protein ZipA, known to
bind the filaments to the lipid surface [16], was oriented on a reconstituted lipid surface to attach the filaments. This in vitro reconstituting approach provides high-resolution structural and dynamic information of the individual filaments on the different surfaces and allows identification of filament interactions responsible for determining the equilibrium structures observed.

2 Materials and Methods

2.1 Reagents

Guanine nucleotides, GDP and GTP, were from Sigma and Roche Molecular Biochemicals, respectively. The nucleotide analog GMPCPP, which hydrolyzes more slowly than GTP, was purchased from Jena Bioscience. Other analytical grade chemicals were from Merck or Sigma.

2.2 Protein Purification and Assay

*Escherichia coli* FtsZ was purified by the calcium-induced precipitation method as described [17]. The protein concentration was measured using the bicinchoninic acid assay (Pierce) with spectrophotometrically calibrated FtsZ standards [17]. ZipA with a histidine tag was prepared as described in [18].

2.3 Atomic Force Microscopy

Atomic force microscope (AFM) images were taken with a microscope from Nanotec Electrónica, Madrid, Spain, operated in the jump mode [19]. The scanning piezo was calibrated using silicon calibrating gratings (NT-MDT Moscow, Russia). Silicon nitride tips (DI instruments) with a force constant of 0.05 N/m were used. A drop of the solution with the FtsZ polymers (formed upon addition of 10 mM GTP to FtsZ protein solutions in Tris 50 mM, pH 7, 0.5 M KCl, 5 mM MgCl₂ buffer) was incubated over freshly cleaved circular pieces of mica glued onto a Teflon surface. After a few minutes, the protein solution was removed and samples were extensively washed with working buffer and imaged under buffer solution containing 1 mM GTP. Images of the same area were acquired over a period of more than 1 h. To confirm that the imaging itself was not perturbing the dynamic behavior of the polymers, we analyzed regions away from the previously scanned surface and always observed similar changes (images not shown). At least three different tips were used for the measurements. Formation of AlF₃ in solution was performed by addition of 10 mM NaF to a 100-μM AlCl₃ solution.

2.4 Planar Lipid Bilayers

Planar lipid bilayers were formed by fusing 100 nm diameter LUVs of lipids purchased from Avanti polar lipids (*E. coli* polar extract, egg PC, and DOGS-NTA Ni salt) on a mica surface. For orienting the protein on a first bilayer constituted with 1:100 molar ratio of DOGS-NTA/egg PC, the protein–lipid–detergent ternary complex prepared as described in [20] was incubated overnight in the presence of BioBeads (Bio-Rad).
3 Results

3.1 FtsZ Individual Filaments on Mica

Atomic force images of individual FtsZ filaments adsorbed on a mica surface were obtained. Figure 1 shows two regions with different protein surface coverage. If a large amount of protein is adsorbed on the surface, the individual filaments are densely packed. The structures formed manifest two traits: a high flexibility that allows individual polymers to adopt curved structures and a strong tendency to interact laterally that keeps them arranged in bundles (Fig. 1a). When the GTP present is consumed and the density of the filaments decreases to a point where they can rearrange on the surface, they evolve into spiral structures that maximize the extent of the energetically favored lateral interactions. A more detailed description of the behavior of the FtsZ filaments on a mica surface and their polymerization in solution is presented elsewhere [21, 22].

3.2 Modeling

3.2.1 Short Filaments

In an attempt to identify the monomer interactions responsible for governing the observed structures, we modeled the filaments considering the protein monomers as beads in a chain and with a few parameters to represent their effective interactions (Fig. 2). We first considered an isolated filament formed by \( N \) monomers, constrained to the \( XY \) plane, and linked by spring forces both in the relative distance between adjacent beads and in the relative angle between consecutive bonds, but without any kind of lateral interaction between the beads. The configuration of the filament is given by the coordinates \( r_i = (x_i, y_i) \) of the \( (i = 1 \) to \( N \)) monomers, and the potential energy of each configuration is:

\[
U_0 ([r]) = \frac{\kappa_o}{2} \sum_{i=1}^{N-1} (|r_{i+1} - r_i| - r_o)^2 + \frac{\kappa}{2} \sum_{i=2}^{N-1} (\theta_i - \theta_0)^2
\]  

(1)

where \( \theta_i \) is the angle between the \((i-1, i)\) and the \((i, i+1)\) bonds, \( \kappa \) is the angular spring constant, and \( \theta_0 \) the preferred angle between the monomers.
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**Fig. 2** Modeling of short filaments. **a** Protein filaments are considered as beads on a string kept together by a spring constant \( \kappa \) and a preferential angle \( \theta_0 \). **b** An image of one of the filaments analyzed to obtain the distribution of angles between monomers shown in **c**.

The schematic drawing in Fig. 2a depicts the model and the potential energy used to describe the typical shape of the filaments. An analysis of digitalized images of filaments such as the one depicted in Fig. 2b allows the measurement of the distribution of angles formed between monomers in the filaments. Figure 2c shows the measured angle distribution and indicates that the most frequent value of the angle is \( \theta_0 \approx 2.5^\circ \) with a standard deviation of a similar magnitude. The filaments analyzed quantitatively to extract this information were grown in the presence of GDP and AlF\(_3\) instead of GTP. This compound is a high-affinity analog of the \( \gamma \)-phosphate of GTP at the active site of G proteins and microtubules and inhibits the GTPase activity of FtsZ [23–25]. This allowed us to analyze the shape of stable filaments that did not depolymerize due to GTP hydrolysis.

### 3.2.2 Long Filaments

The lengthening of the filaments by the incorporation of monomers to the ends gave rise to the formation of spirals. In order to model their formation, additional considerations had to be included in the potential energy description. Adding a heuristic Lennard–Jones bead–bead interaction to the ideal chain energy given by Eq. 1 that accounts for the steric
repulsions, which forbid chain crossings, and the lateral attractions, which promote the formation of the spirals, gives rise to the following potential:

$$U([r]) = U_0([r]) + 4\varepsilon \sum_{ij}^{N} \left[ \left( \frac{\sigma}{r_{ij}} \right)^{12} - \left( \frac{\sigma}{r_{ij}} \right)^{6} \right].$$

(2)

The relevant parameters are the distance between filaments $\sigma$, for the repulsive core between monomers, and the depth of the attractive potential well, $\varepsilon$. $\sigma$ may be directly estimated from AFM profiles of higher-resolution images of similar coiled structures [21], and $\varepsilon$ can be estimated experimentally through its effects in establishing the inner and outer radius of the equilibrium coils.

Figure 3 depicts schematically the adequacy of the model to reproduce the images observed. Figure 3a shows images of the same FtsZ filaments taken at different times. The shorter filaments (left hand side), curved when short, adopt an equilibrium spiral structure (right hand side) when they lengthen. The potential presented in Eq. 2 is enough to allow for the formation of spiral structures when used in Langevin computer simulations to explore the equilibrium configurations adopted by extended filaments diffusing on a two-dimensional surface (Fig. 3c). Quantitative analysis of the AFM images allows estimation of the bending modulus and the lateral attraction between filaments. These values indicate

**Fig. 3** Modeling of long filaments. a Images of the same filaments taken at different times. Shorter filaments on the left lengthen to form the spirals shown at the right. b The two drawings are taken from the Langevin dynamic simulations run using the potential shown in b (Eq. 2) to explore the equilibrium configurations adopted by extended filaments diffusing on a two-dimensional surface
that there is a close balance between the flexural energy and the optimization of the lateral contacts in the FtsZ filaments. The complete description of the theoretical modeling outlined above can be found in reference [26].

3.3 FtsZ Filaments on Lipid Surfaces

It was of interest to explore the behavior of the filaments on surfaces mimicking the natural lipid environment instead of using a nonbiologically relevant surface as mica. FtsZ ring formation on the inner surface of the cytoplasmic membrane requires the presence of membrane-anchoring proteins. Two proteins have been described to play this role: ZipA and FtsA [16, 27]. ZipA is a membrane protein with an N-terminal transmembrane domain, a flexible linker, and a carboxyl terminal FtsZ binding domain [28]. We, therefore, attempted to orient the asymmetric protein ZipA on a lipid-supported bilayer adapting a protocol previously described to form two-dimensional crystals of membrane proteins on lipid monolayers [20]. Figure 4 depicts a cartoon summarizing the protocol used to orient the histidine-tagged E. coli native ZipA protein on a supported bilayer containing lipids with NTA binding sites. This bilayer with oriented proteins is then exposed to the FtsZ polymers formed in solution in the presence of GTP. Figure 5a, b shows the lack of affinity of the filaments for pure lipid bilayers. Figure 5a represents a mica surface (darker region) partially covered by a lipid bilayer. Figure 5b shows the same surface after incubation

Fig. 4 Orientation of native ZipA on a planar lipid bilayer. a Formation of the first supported membrane on a mica surface. b and c The step in which the protein–lipid–detergent solubilized complex is incubated in the presence of BioBeads to remove excess detergent. The histidines in the amino terminal end of the ZipA bind to the first bilayer and orient the protein, while removal of the detergent allows for the formation of a second bilayer in which the protein is incorporated. d The surface that can then be exposed to the FtsZ protein solution and analyzed with the AFM tip
Fig. 5  a  A mica surface (darker region) partially covered by a lipid bilayer. b The same surface after incubation with the protein. FtsZ filaments adsorb to the bare mica but not to the lipid surface. c The surface of E. coli lipids with ZipA after being exposed to FtsZ in the presence of GTP. d The same region 15 min later, after the formation of FtsZ filament bundles

with the protein. The affinity of the filaments for the mica is much larger than for the lipid surface. The protein readily binds to the mica, avoiding the bare lipid surfaces. However, once ZipA is incorporated into the bilayer (Fig. 5c, d), FtsZ adsorbs to the lipid surfaces. Figure 5c shows the lipid surface a few minutes after the addition of the proteins. After several minutes, protein aggregates are observed to fuse and align, forming bundles whose thickness corresponds to that of five to 15 individual filaments. It is interesting to note that this surface polymerization was only observed when the ZipA protein was reconstituted in the E. coli lipid polar extract. Reconstitution using other lipids resulted in the formation of surface patches of ZipA that did not permit the formation of bundles after incubation with the FtsZ protein in the presence of GTP.

The filaments do not attach as such to the lipid surface. It takes several minutes until the FtsZ and the ZipA found on the surface copolymerize giving rise to the observed bundles. The bundling activity of ZipA has been previously described [29].

4 Discussion

The present work provides experimental data on the structure and dynamic behavior of FtsZ filaments on different surfaces. The AFM allowed observation of the shape and time evolution of individual filaments [21]. This high-resolution information was then modeled to estimate the values of relevant parameters determining their shape: preferential angle between monomers in the filaments, the bending modulus, and the lateral attraction between filaments. A preferential angle $\theta_0 \approx 2.5^\circ$ with a standard deviation of similar magnitude is present even when GTP is bound to the monomers, contrary to the previous suggestion that the curved conformation was associated with the presence of GDP [30, 31].

The simulations indicated that the joint effect of a preferential curvature and a low flexural rigidity lead to a crucial effect of the lateral attractions in the typical shapes
of filaments larger than 0.5 μm. The estimated value of the attractive energy per lateral attraction is around $\epsilon = 5 \text{ kJ/mol}$, equivalent to the energetic cost of bending a bond $4^\circ$ away from its preferential angle. That gives a clear picture of the close balance between the flexural energy and the optimization of the lateral contacts. The filaments can, therefore, be considered as “linear aggregates” with a hierarchical structure in which the lateral attractions are important, but significantly weaker than the bond energy between monomers.

Our analysis points to the importance of lateral interactions between filaments in determining their equilibrium structures. Furthermore, using the model to explore the behavior of the filaments on a curved surface indicates that sufficiently long filaments would wrap around the cylinder. The same energetically favorable condition, maximization of lateral interactions, which leads to the formation of spirals on a flat surface, would, in this case, act to decrease the radius of the FtsZ ring, thus generating the constriction force necessary for cell division [26, 32]. Although further experiments are required to understand why these interactions are relevant to the function of the polymers in vivo, our results suggest that they could be the basis of a plausible force-generating mechanism and offer an explanation for previous experimental evidence associating alterations in the lateral amino acids with impaired in vivo function [33].

The quantitative estimation of parameters such as preferential angle, lateral attraction, and bending modulus from experimental data is scarce for bacterial polymers [34]. The data provided in this analysis are, therefore, important for the refinement of proposed models constructed extrapolating quantitative data obtained from eukaryotic polymers [32, 35].

FtsZ filaments attached to a lipid surface through the interaction with oriented ZipA reconstituted on *E. coli* lipid bilayers present a different morphology to that observed on the mica surface. Although more work needs to be done in order to understand the exact role of the anchoring protein ZipA in remodeling the individual filaments, it is clear that it not only retains the filaments near the surface but also affects the interactions among the filaments inducing the formation of higher-order structures. This bundling effect of ZipA had been observed in solution [29], but the availability of an assay that allows the observation in real time with enough spatial resolution to distinguish individual filaments and their evolution into higher-order structures will help answer important questions. The use of mutants will help determine which ZipA domains are relevant for attaching and bundling the FtsZ filaments and to contrast the proposed mechanisms by which the membrane interactions of the filaments constrain or modulate the possible force-generating mechanism [32, 36].

In summary, we have shown that individual FtsZ filaments can adopt different structures on a mica surface. Theoretical modeling of the structures observed has allowed the extraction of quantitative information about the polymers and the interactions that determine their shapes. Reconstituting the membrane-anchoring protein ZipA on an *E. coli* lipid membrane on surfaces provides information on how membrane attachment regulates polymer dynamics and indicates the important role played by the lipid composition of the membrane. Understanding the interactions between these cell division components will help control their assembly in other artificial systems such as giant liposomes that more closely mimic the curved membrane surface of the bacteria.

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