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Immediate GTP hydrolysis upon FtsZ polymerization

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Summary
To understand the polymerization dynamics of FtsZ, a bacterial cell division protein similar to tubulin, insight is required into the nature of the nucleotide bound to the polymerized protein. In a previous study, we showed that the FtsZ polymers contain mostly GDP. A recent study challenged this result, suggesting that the polymerized FtsZ is in a GTP-bound state. Here, we show that, when radiolabelled [γ-32P]-GTP is used to polymerize FtsZ, GTP is hydrolysed instantaneously. The FtsZ polymer contains both GDP and the radiolabelled inorganic phosphate.

Introduction
FtsZ is a key prokaryotic cell division protein, forming a structural element known as the Z-ring at the site of cell division (reviewed by Margolin, 2000; Scheffers and Driessen, 2001). In Escherichia coli, the Z-ring is critical for the localization of all other protein components of the cell division machinery. The Z-ring is likely to consist of polymers similar to the FtsZ polymers that can be formed in vitro in a GTP-dependent manner (Bramhill and Thompson, 1994; Erickson et al., 1996; Mukherjee and Lutkenhaus, 1998). FtsZ is a 40.3 kDa protein that binds and hydrolyses GTP (De Boer et al., 1992; RayChaudhuri and Park, 1992; Mukherjee et al., 1993). It shows a striking structural similarity to α- and β-tubulins, both as a monomer and when modelled onto protofilaments (Löwe and Amos, 1998; 1999; Nogales et al., 1998; 1999). FtsZ and tubulins share a unique GTP-binding motif, the G-box [GGGTG(ST)G] (De Boer et al., 1992).

Structural modelling of the FtsZ crystal structure onto electron microscopy images of the FtsZ protofilament strongly indicated that the active site for GTP hydrolysis is shared between two FtsZ subunits. In this organization, the GTP-binding pocket is provided by one subunit, while the GTPase-activating T7 loop that could be involved in Mg2+ co-ordination comes from the other subunit (Löwe and Amos, 1999). This structural organization is similar to that of the α,β-tubulin heterodimer in microtubules (Nogales et al., 1999). Work in our laboratory provided biochemical evidence in favour of this model: first, a critical aspartate residue in the T7 loop is involved in cation co-ordination (Scheffers et al., 2001) and, secondly, several T7 loop mutants inhibited in GTP hydrolysis suppress the GTPase activity of wild-type FtsZ when mixed (Scheffers et al., 2002).

With the active site for GTP hydrolysis formed upon association of FtsZ monomers, it is likely that GTP hydrolysis closely follows FtsZ polymerization. Various reports have shown that GTPase activity of FtsZ occurs only at FtsZ concentrations that allow the formation of large FtsZ polymers (Wang and Lutkenhaus, 1993; Lu et al., 1998; Mukherjee and Lutkenhaus, 1998; Sossong et al., 1999). This is indicative of self-activation of the FtsZ GTPase activity: association of FtsZ monomers triggers the GTPase activity of FtsZ. However, several other reports describe the non-co-operative formation of FtsZ protofilaments (Rivas et al., 2000; 2001; Romberg et al., 2001) with GTP hydrolysis predicted to lag behind polymerization.

One of the major questions in the study of FtsZ polymerization is the state of the nucleotide bound to the FtsZ polymer. Previously, we reported that the FtsZ polymer predominantly contains GDP (Scheffers et al., 2000). However, Mingorance et al. (2001) have recently presented results from which it was concluded that FtsZ polymers formed in solution mostly contain GTP. Combined with nucleotide exchange experiments, it was concluded that, during maximal FtsZ polymerization, GDP is not a significant fraction of the bound nucleotide and that nucleotide exchange can occur on all polymer subunits, irrespective of the nature of the bound nucleotide. These conclusions contradict our previous findings. Here, we provide further evidence to show that the FtsZ polymer contains GDP and that the inorganic phosphate formed upon the hydrolysis of GTP is retained by the polymer.

Results
Nucleotide content of FtsZ at limiting GTP concentrations
Briefly, Mingorance et al. (2001) described experiments in
which radiolabelled GTP was added to FtsZ, after which the amount of FtsZ-bound nucleotide was determined by separation of the protein-bound nucleotide from free nucleotide by binding of the protein to nitrocellulose filters. [α-32P]-GTP was used to determine the total nucleotide (GTP+GDP) content of the protein, whereas [γ-32P]-GTP was used to determine the amount of GTP bound to the protein. As retention of radioactivity with [γ-32P]-GTP is represented as GTP bound to FtsZ, the interpretation of the experiment is based on the assumption that, after GTP hydrolysis, the release of inorganic phosphate is very rapid. The presence of a small fraction of the protein containing bound GDP+P is not ruled out but, without quantitative data, it is questionable as to whether this really only concerns a small fraction.

We have carried out similar experiments, but used thin-layer chromatography (TLC) to identify the label bound to protein. For this purpose, FtsZ was incubated with different radiolabelled nucleotides under specified conditions, and the protein was quickly recovered from the solution by ammonium sulphate precipitation. Nucleotides bound to the protein fraction were extracted using perchloric acid and analysed by TLC. We used limiting concentrations of GTP to prevent significant exchange of fresh nucleotide, which would make the results more difficult to interpret (see below). The amount of GTP used is sufficient to allow the formation of polymers in the presence of 5 mM Mg²⁺ and 10 mM Ca²⁺, as was shown previously by both light scattering and electron microscopy (Scheffers et al., 2000). FtsZ that is incubated in cation-containing buffers (Mg²⁺, Ca²⁺ or both) immediately hydrolyses GTP upon addition of [α-32P]-GTP or [γ-32P]-GTP (Fig. 1). When Ca²⁺ is the only cation present, a significant portion of the GTP is not hydrolysed immediately and recovered as [γ-32P]-GTP bound to FtsZ (Fig. 1A, lane 2). However, as soon as Mg²⁺ is included, practically all the FtsZ-bound nucleotide is GDP (Fig. 1B, lanes 3 and 4). When [γ-32P]-GTP is used, hydrolysed 32P co-precipitates with the protein fraction indicating that it remains bound to FtsZ (Fig. 1A, lanes 3 and 4). This demonstrates that the radioactivity retained by filtration of the FtsZ incubated with [γ-32P]-GTP observed by Mingorance et al. (2001) must represent radiolabelled inorganic phosphate. As radiolabelled inorganic phosphate is also retained after hydrolysis in the absence of Ca²⁺ (Fig. 1A, lane 3), we exclude the possibility that the spots are the result of Ca₃(PO₄)₂ precipitation. Moreover, the perchloric acid extraction of the bound nucleotide did not induce the hydrolysis of GTP, as GTP-bound FtsZ could be recovered when the protein was incubated with GTP in the presence of the chelators EDTA and EGTA to prevent hydrolysis (Fig. 1A and B, lane 5). When FtsZ was replaced by an unrelated protein, i.e. bovine serum albumin (BSA), no nucleotide could be recovered in the pellet fraction after ammonium sulphate precipitation (Fig. 1A and B, lane 6).

Nucleotide content of FtsZ at saturating GTP concentrations

To ensure that the observed retention of inorganic phosphate is also valid at higher GTP concentrations that lead to significant polymerization in the presence of cations (Mukherjee and Lutkenhaus, 1999), the experiments were repeated with 1 mM GTP. Moreover, to validate the ammonium sulphate precipitation assay, we used both precipitation (Scheffers et al., 2000) and filtration (Mingorance et al., 2001) to analyse the nucleotide contents of the polymers. For this purpose, the nucleotide retained on the filter was immediately extracted with ice-cold perchloric acid and analysed by TLC. Even when polymer mixtures were filtered immediately after the addition of label, a significant GTP hydrolysis and retention of radiolabelled inorganic phosphate was observed (Fig. 2). Identical results were obtained when the FtsZ was recovered by ammonium sulphate precipitation (not shown). These data also demonstrate that, at high GTP concentrations, the FtsZ polymer mostly contains bound GDP.

Discussion

In the description of models for FtsZ polymerization, the kinetics of nucleotide hydrolysis, release and/or exchange play a pivotal role. Previously, we have shown that FtsZ polymers containing mostly GDP can be stabilized by GTP-γ-S (Scheffers et al., 2000), suggesting that FtsZ...
The observed rapid exchange of bound radiolabelled nucleotides on the polymer with added GTP (Mingorance et al., 2001) does not reflect the exchange of FtsZ-bound GTP, but FtsZ-bound GDP with GTP from solution. Further, the model for isodesmic FtsZ polymerization, in which most of the FtsZ subunits in the polymer contain GTP (Romberg et al., 2001), seems unlikely given our biochemical data. The immediate GTP hydrolysis we describe here fits a model in which the active site for GTP hydrolysis is formed by the contact between two FtsZ monomers, leading to immediate hydrolysis of the bound GTP upon FtsZ–FtsZ association (reviewed by Scheffers and Driessen, 2001). Previously, we have presented evidence pointing out that the stability of FtsZ polymers may be defined by the presence of a ‘GTP cap’ as described for tubulin (Scheffers et al., 2000). It remains to be determined whether this cap consists of GTP-bound FtsZ or GDP-Pi-bound FtsZ. The organization of microtubules suggests that the microtubule plus end is always capped by at least one GTP-bound tubulin heterodimer. Whether microtubules are stabilized by GDP-P, is, however, still a matter of debate (reviewed by Nogales, 2000). Further polymer stability could be provided by the rapid exchange of GDP for GTP within the polymer (Mingorance et al., 2001). More evidence for co-operative assembly of FtsZ polymers and stabilization of the FtsZ polymer by a GTP cap was described recently by Mukherjee et al. (2001). In this report, we show that GTP is hydrolysed immediately by FtsZ when added to FtsZ at polymerizing conditions, with retention of P. The role of P, release in the polymerization of FtsZ has hardly been addressed to date, with most reports in the literature solely considering GTP- or GDP-bound FtsZ. Recently, it has been shown that the γ-phosphate of GTP in FtsZ is sensed by the T3 loop, similar to tubulin (Diaz et al., 2001). Molecular dynamic studies using the FtsZ crystal structure showed that GTP-bound FtsZ has a more compact conformation than GDP-bound FtsZ, and P, release may have a decisive impact on polymer conformation and stability (Diaz et al., 2001). We have obtained evidence that a conserved positive charge, located in the T7 loop region of FtsZ, may also be implicated in co-ordination of the binding and release of P, after GTP hydrolysis (Scheffers et al., 2002). Combined with the data in this report, further studies on the dynamics of P, retention and release from the FtsZ polymer are required.

**Experimental procedures**

**Materials**

FtsZ was purified as described previously (Scheffers et al., 2000), and the FtsZ concentration was determined using a Bradford assay with a correction factor of 0.82 for the FtsZ:BSA ratio (Lu et al., 1998). \([\gamma^{32}\text{P}]-\text{GTP} (3000 \text{ Ci mmol}^{-1})\)
and [γ-32P]-GTP (5000 Ci mmol⁻¹) were obtained from Amersham Pharmacia Biotech. Other chemicals were obtained from Sigma-Aldrich.

**Analysis of FtsZ-bound nucleotides by TLC**

FtsZ (0.5 mg ml⁻¹) was incubated at 30°C in 50 mM Mes/NaOH (pH 6.5), 50 mM KCl (buffer A) and cations when indicated. Either [γ-32P]-GTP or [α-32P]-GTP was added to the concentrations indicated, and the FtsZ was recovered by filtration or ammonium sulphate precipitation. In the case of the filtration assay, the FtsZ–GTP mixtures (20 μl) were filtered through nitrocellulose disks (pore size 45 μm; Whatman) and washed with 400 μl of wash buffer [20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgSO₄, 0.005% Triton X-100, 20 mM (NH₄)₂SO₄] as described previously (Mingorance et al., 2001). Subsequently, the filters were extracted using 50 μl of ice-cold perchloric acid solution (a 1:2 v/v mixture of 14% perchloric acid, 9 mM EDTA and buffer A). The pH of the samples was adjusted using 25 μl of 1 M KHCO₃, 1 M KOH, and the samples were clarified by centrifugation for 3 min at 20 000 g. Samples of 10 μl were spotted on CEL 300 PEI/UV254 plates (Macherey Nagel) and analysed by TLC using 0.65 M KH₂PO₄/H₃PO₄, pH 3.5, as running buffer. Plates were dried and exposed to Kodak Biomax MR films. For the precipitation assay, the FtsZ–GTP mixture (20 μl) was precipitated using 20 μl of a 75% (w/v) mixture (20 μl of l of ice-cold perchloric acid solution; <10 s). Precipitation was performed on Kodak Biomax MR films. For the precipitation assay, the FtsZ–GTP mixture (20 μl) was precipitated using 20 μl of a 75% (w/v) ammonium sulphate solution in buffer A immediately upon addition of GTP (handling time between the addition of GTP and precipitation was <10 s). Precipitation was performed on ice for 10 min, and the protein was recovered by centrifugation for 5 min at 20 000 g. Subsequently, nucleotides bound to FtsZ were extracted using perchloric acid and analysed by TLC as described above.

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