

# ***Escherichia coli* FtsZ polymers contain mostly GTP and have a high nucleotide turnover**

Jesús Mingorance, Sonsoles Rueda, Paulino Gómez-Puertas, Alfonso Valencia and Miguel Vicente\*

Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco, 28049 Madrid, Spain.

## **Summary**

The cell division protein FtsZ is a GTPase structurally related to tubulin and, like tubulin, it assembles *in vitro* into filaments, sheets and other structures. To study the roles that GTP binding and hydrolysis play in the dynamics of FtsZ polymerization, the nucleotide contents of FtsZ were measured under different polymerizing conditions using a nitrocellulose filter-binding assay, whereas polymerization of the protein was followed in parallel by light scattering. Unpolymerized FtsZ bound 1 mol of GTP mol<sup>-1</sup> protein monomer. At pH 7.5 and in the presence of Mg<sup>2+</sup> and K<sup>+</sup>, there was a strong GTPase activity; most of the bound nucleotide was GTP during the first few minutes but, later, the amount of GTP decreased in parallel with depolymerization, whereas the total nucleotide contents remained invariant. These results show that the long FtsZ polymers formed in solution contain mostly GTP. Incorporation of nucleotides into the protein was very fast either when the label was introduced at the onset of the reaction or subsequently during polymerization. Molecular modelling of an FtsZ dimer showed the presence of a cleft between the two subunits maintaining the nucleotide binding site open to the medium. These results show that the FtsZ polymers are highly dynamic structures that quickly exchange the bound nucleotide, and this exchange can occur in all the subunits.

## **Introduction**

The FtsZ protein is a ubiquitous element in the cell division of prokaryotes. It is found in most eubacteria and archaea, and also in chloroplasts and some mitochondria (Rothfield *et al.*, 1999; Beech *et al.*, 2000; Erickson, 2000). In *Escherichia coli*, it is an essential protein; it is relatively abundant and is found preferentially in the cytoplasm (Pla

*et al.*, 1991), but can also be isolated from the inner cell membrane (Jones and Holland, 1985). During cell division, FtsZ localizes into a ring at the division site, the Z-ring, that seems to constrict as the septum grows inward (reviewed by Bramhill, 1997; Lutkenhaus and Addinall, 1997; Rothfield *et al.*, 1999). Although the exact role that FtsZ plays in septal peptidoglycan synthesis and cell constriction is not yet known, it is required for the proper localization of several other cell division proteins in *E. coli* and *Bacillus subtilis*, among them PBP3 (= FtsI), the penicillin-binding protein involved in the synthesis of septal peptidoglycan (reviewed by Vicente and Errington, 1996).

FtsZ is structurally related to tubulin (Löwe and Amos, 1998; Nogales *et al.*, 1998). It has a strong GTPase activity (de Boer *et al.*, 1992; RayChaudhuri and Park, 1992; Mukherjee *et al.*, 1993; Lu *et al.*, 1998), which is dependent on magnesium and on the concentration of FtsZ, suggesting that the enzymatic activity is related to a self-association process (Mukherjee and Lutkenhaus, 1994; Sossong *et al.*, 1999). *In vitro*, it self-associates forming linear oligomers in a nearly isodesmic process driven by magnesium, which is dependent on the concentration of the protein and the ionic strength (Rivas *et al.*, 2000). Recently, it has been proposed that assembly in the presence of GTP is also isodesmic and non-co-operative (Romberg *et al.*, 2001). Upon addition of several promoting agents, the protofilaments associate forming tubes, rings and other structures (Bramhill and Thompson, 1994; Erickson *et al.*, 1996; Mukherjee and Lutkenhaus, 1994; 1998; Trusca *et al.*, 1998; Löwe and Amos, 1999; 2000; Lu *et al.*, 2000). Several models have been proposed to relate these properties of FtsZ with cell division (Bramhill, 1997; Erickson, 1997); however, the mechanisms that relate GTP binding, GTP hydrolysis and self-association and the roles that these processes play in FtsZ function are not yet known.

Desai and Mitchison (1998) have proposed two possible models to relate the modes of polymerization and hydrolysis with their functional implications. If the nucleotide is non-exchangeable in the polymer, the resulting protofilaments might be tubulin-like and contain a GDP core with GTP caps at one or both ends. In these polymers, GTP hydrolysis might drive polymerization dynamics as in tubulin. Alternatively, the nucleotide might be exchangeable along the polymer independently of polymer assembly or disassembly processes. In this case, the role of GTP hydrolysis might be to drive changes in the shape of the

Accepted 23 April, 2001. \*For correspondence. E-mail mvicente@cib.uam.es; Tel. (+34) 91 585 46 99; Fax (+34) 91 585 45 06.

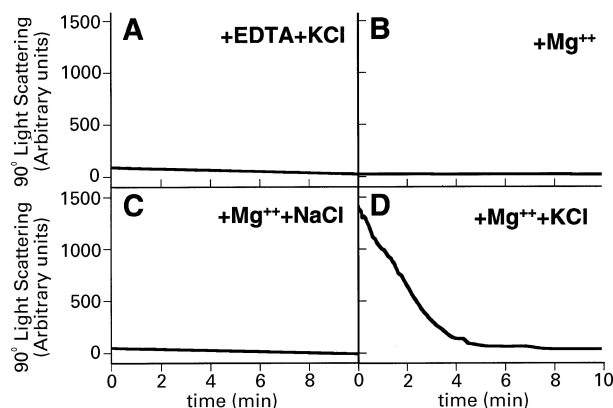
filaments. In the case of FtsZ, the first model seems to be favoured by some structural and biochemical data obtained when studying polymerization in the presence of  $\text{Ca}^{2+}$  (Löwe and Amos, 1999; Scheffers *et al.*, 2000), whereas the second model might be supported by the finding that GTP favours a straight conformation, while GDP favours a curved conformation for the filament (Lu *et al.*, 2000; Díaz *et al.*, 2001).

In this work, we have studied the nucleotide composition of FtsZ and the dynamics of nucleotide exchange in different conditions. Our results show that, in the absence of stabilizing agents, such as  $\text{Ca}^{2+}$ , or DEAE-dextran, the nucleotide bound to FtsZ polymers is mostly GTP and, moreover, the bound nucleotide is exchanged very fast. Thus, *in vitro*, the nucleotide is exchangeable along the polymer, suggesting that FtsZ works by a mechanism different from that of tubulin, and that FtsZ itself might have a motor role in cell division.

## Results

### Polymerization of FtsZ at pH 7.5 depends on $\text{Mg}^{2+}$ and $\text{K}^+$

Light scattering spectroscopy shows that, at pH 6.5, *E. coli* FtsZ polymerizes even in the absence of  $\text{Mg}^{2+}$  and  $\text{K}^+$  cations (not shown), as reported previously by other groups (Lu *et al.*, 1998; Mukherjee and Lutkenhaus, 1999). However, the intracellular pH is well regulated in *E. coli*, ranging from 7.4 to 7.8 over an extracellular pH range between 5.5 and 9 (Slonczewski *et al.*, 1981). To study the activity of FtsZ under more physiological conditions, we analysed polymerization and hydrolysis at pH 7.5 (Fig. 1). In the presence of 10 mM EDTA and without added  $\text{Mg}^{2+}$ , the addition of GTP, to a concentration of 0.5 mM, did not induce polymerization (Fig. 1A), and there was no



**Fig. 1.** Cation dependence of FtsZ polymerization at pH 7.5. FtsZ (10  $\mu\text{M}$ ) was incubated at 25°C in 50 mM Tris, pH 7.5, in the presence of (A) 10 mM EDTA and 100 mM KCl, (B) 5 mM  $\text{MgCl}_2$ , (C) 5 mM  $\text{MgCl}_2$  and 100 mM NaCl, (D) 5 mM  $\text{MgCl}_2$  and 100 mM KCl. GTP was added to a concentration of 0.5 mM, and polymerization was followed by 90° light scattering in a fluorimeter.

detectable GTPase activity (Table 1, first row). The same was observed in the presence of 5 mM  $\text{Mg}^{2+}$ , without monovalent salts (Fig. 1B) or with 100 mM NaCl (Fig. 1C). Only when both  $\text{Mg}^{2+}$  and  $\text{K}^+$  were included in the reactions were GTP-dependent polymerization (Fig. 1D) and GTPase activity observed (Table 1, third row). In this case, polymerization was followed by rapid depolymerization as a result of GTP consumption.

Electron microscopy of the polymers formed at pH 7.5 in the presence of  $\text{Mg}^{2+}$  and  $\text{K}^+$  confirmed the formation of long FtsZ filaments, many of them several micrometres long (Fig. 2A), formed by two, three or more protofilaments paired along their longer axis (Fig. 2B).

### Nucleotide contents of FtsZ polymers

The nucleotide contents of FtsZ were measured using radiolabelled GTP followed by binding of the protein to nitrocellulose filters to separate the protein-bound from the free nucleotide. As this procedure does not differentiate between polymeric and non-polymeric protein, the polymerization of FtsZ was analysed in parallel reactions by light scattering and electron microscopy.

To measure the nucleotide contents of the protein, radioactively labelled GTP was added to a concentration of 0.5 mM (0.4 Ci  $\text{mmol}^{-1}$ ), the samples were filtered through nitrocellulose, and the radioactivity retained in the filters was measured in a scintillation counter. In the presence of EDTA, the total nucleotide contents of the protein were  $0.86 \pm 0.10$  mol  $\text{mol}^{-1}$  FtsZ 1 min after the addition of [ $\alpha$ - $^{32}\text{P}$ ]-GTP and remained stable for several minutes. As there was no GTPase activity, all the nucleotide bound to the protein should be GTP. This was confirmed by measuring the binding of [ $\gamma$ - $^{32}\text{P}$ ]-GTP, which yielded  $0.92 \pm 0.09$  mol of GTP bound  $\text{mol}^{-1}$  FtsZ.

To study the nucleotide contents of FtsZ polymers, the assays were performed at pH 7.5 in the presence of 5 mM  $\text{Mg}^{2+}$  and 100 mM  $\text{K}^+$ . Under these conditions, the GTPase activity was strong (turnover rate  $6.5 \pm 0.7$   $\text{min}^{-1}$ ; Table 1); therefore, the GTP was quickly depleted and the protein filaments depolymerized (Fig. 1D). Increasing the concentration of radiolabelled GTP to 2 mM (0.25 Ci  $\text{mmol}^{-1}$ ) extended the time needed to exhaust GTP, and therefore increased the life of the polymers (Fig. 3A). Owing to the GTPase activity of FtsZ, the samples contained both nucleotides in this case, GTP and GDP. Therefore, [ $\alpha$ - $^{32}\text{P}$ ]-GTP was used for measuring the total nucleotide (GDP + GTP) contents of the protein, and [ $\gamma$ - $^{32}\text{P}$ ]-GTP for measuring only GTP. The total nucleotide contents remained constant throughout the course of the experiment, with an average of  $1.1 \pm 0.06$  mol of nucleotide  $\text{mol}^{-1}$  FtsZ. The amount of bound GTP was  $1.0 \pm 0.1$  mol of GTP  $\text{mol}^{-1}$  FtsZ 15 s after the onset of the reaction; it remained at approximately 1 mol of GTP

**Table 1.** Nucleotide exchange and GTPase activities of FtsZ polymers in the presence of  $Mg^{2+}$  and  $Ca^{2+}$ .

| [ $Mg^{2+}$ ]<br>(mM) | [ $K^+$ ]<br>(mM) | [ $Ca^{2+}$ ]<br>(mM) | Nucleotide exchange turnover<br>( $min^{-1}$ ) | GTPase turnover<br>( $min^{-1}$ ) |
|-----------------------|-------------------|-----------------------|--|-----------------------------------|
| –                     | 100               | –                     | $\geq 4.16^a$                                  | 0                                 |
| 5                     | 25                | –                     | $\geq 4.16^a$                                  | $3.2 \pm 0.4$                     |
| 5                     | 100               | –                     | $\geq 4.16^a$                                  | $6.5 \pm 0.7$                     |
| 5                     | 25                | 10                    | $1.20 \pm 0.64$                                | $0.20 \pm 0.04$                   |
| 5                     | 50                | 10                    | $1.08 \pm 0.30$                                | $0.62 \pm 0.10$                   |
| 5                     | 100               | 10                    | $1.40 \pm 0.38$                                | $1.29 \pm 0.11$                   |

Polymerization was induced with 0.5 mM unlabelled GTP and, after 1 min, 0.5  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]-GTP was added. All the experiments were performed at 25°C in 50 mM Tris, pH 7.5, 0.1 mM EDTA. The concentration of FtsZ was 10  $\mu$ M. The experiment shown in the first row contained 10 mM EDTA. The nucleotide contents of the protein were analysed by nitrocellulose filter binding, and the GTPase activity by thin-layer chromatography.

a. Semi-quantitative estimate calculated assuming that half-maximal binding occurs in less than 10 s (see *Experimental procedures*).

$mol^{-1}$  FtsZ for several minutes and then gradually decreased in parallel with the decrease in light scattering (Fig. 3B). This indicated that, as total GTP was depleted, the GTP in FtsZ was substituted by GDP. Although the filter-binding assay does not separate polymers from monomers and oligomers, when the light scattering signal was maximal, the protein-bound nucleotide was mostly GTP, indicating that the FtsZ polymers in which their GTPase was active also contained mainly GTP-FtsZ.

#### Nucleotide exchange in FtsZ polymers

The addition of labelled GTP to the reaction mixtures always resulted in a very fast incorporation of the nucleotide to the protein, under both polymerizing and non-polymerizing conditions. This could result from incorporation of the label to FtsZ monomers before they start polymerizing, and therefore might not reflect the nucleotide dynamics of the polymers.

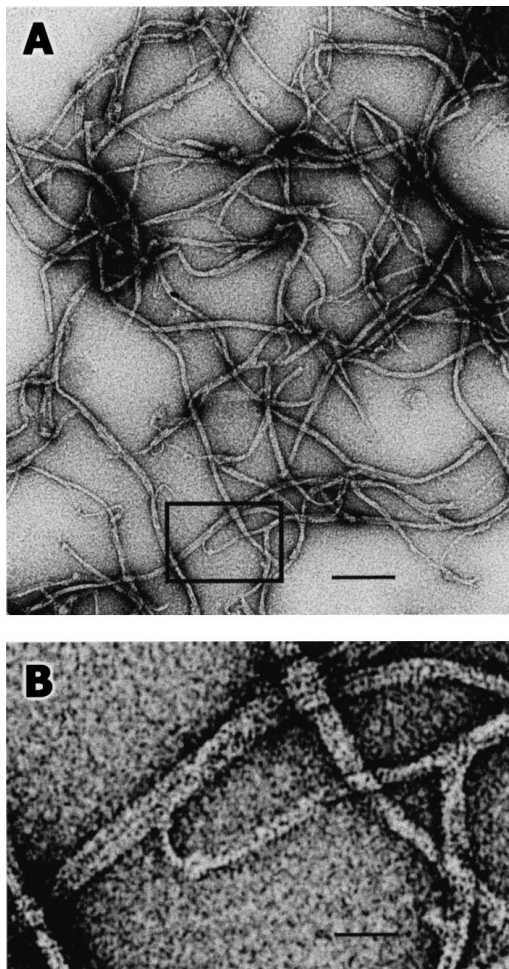
To measure the occurrence and extent of nucleotide exchange in FtsZ, the reactions were started by adding unlabelled GTP to a final concentration of 0.5 mM. After 1 min, 0.5  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]-GTP were added, and the binding of the labelled nucleotide was followed using the nitrocellulose filter-binding assay. Under non-polymerizing conditions (Tris, pH 7.5, 10 mM EDTA, no  $Mg^{2+}$ ), the incorporation of radioactive label to the protein was already maximal in 10 s, showing that, in non-polymeric FtsZ, the GTP binding site is completely accessible to the medium, and the protein continuously exchanged the nucleotide. The exchange was too fast to be measured quantitatively with this technique; a very conservative (and semi-quantitative) estimate gave a turnover rate of  $4.16 min^{-1}$  or higher (see *Experimental procedures*; Table 1).

When the labelled nucleotide was added after 1 min of polymerization in Tris, pH 7.5, in the presence of 5 mM  $Mg^{2+}$  and 100 mM  $K^+$  (Fig. 4, open circles), the incorporation of the radioactive label was again maximal in 10 s, and close to 1 mol of nucleotide bound  $mol^{-1}$  FtsZ

(after correcting for the GTP hydrolysed during the first minute). In this case, the polymers had a high GTPase activity and, therefore, the fast incorporation of the label to the protein might be the result of a high FtsZ monomer turnover through the polymers driven by GTP hydrolysis. The hydrolysis rate should be higher than or equal to the nucleotide exchange rate. Alternatively, it might indicate that all the FtsZ subunits freely exchange nucleotide with the medium and, in this case, the exchange might be faster than the hydrolysis. To distinguish between these two possibilities, the nucleotide exchange and hydrolysis rates were compared in different conditions. The GTP hydrolysis rate was modulated by varying the concentration of KCl in the reaction, but this had no significant effect on the nucleotide exchange, which was the same as in the absence of GTPase activity (Table 1). This indicates that nucleotide exchange and hydrolysis were not coupled in *E. coli* FtsZ polymers, and the fast nucleotide exchange rate found in these experiments was most probably the result of exchange in all the polymer subunits.

To confirm this point further, we next studied the exchange of nucleotide by FtsZ in the presence of 5 mM  $MgCl_2$  and 10 mM  $CaCl_2$ . In this case, the addition of GTP induced a very fast increase in the light scattering signal, which was already maximal in about 10 s (not shown). The signal was higher than in the absence of  $Ca^{2+}$ , indicating the formation of large filament bundles. The GTPase activity was inhibited (Table 1). The incorporation of radioactive label to the protein was immediate when the labelled nucleotide was added at the onset of the reaction, as happened with  $Mg^{2+}$  alone. But, when the tracer nucleotide was added after 1 min of polymerization in the presence of unlabelled GTP (Fig. 4, closed circles), the incorporation of radioactivity to the protein was much slower than in the absence of  $Ca^{2+}$ , reaching 1 mol of nucleotide bound  $mol^{-1}$  FtsZ after 2 min (corrected for GTP hydrolysis before label addition). As before, the nucleotide exchange and hydrolysis rates were measured at several KCl concentrations, and it was found that the nucleotide exchange was constant and independent of  $K^+$ .





**Fig. 2.** Electron microscopy of FtsZ polymers. FtsZ (10  $\mu$ M) was incubated at 25°C in 50 mM Tris, pH 7.5, 100 mM KCl, 0.1 mM EDTA and 5 mM MgCl<sub>2</sub>. GTP was added to a concentration of 0.5 mM and, after 1 min, the protein was adsorbed to collodion-coated sample grids and negatively stained with uranyl acetate. B. A magnification of the area inside the square in (A). The bar represents 200 nm in (A) and 50 nm in (B).

The GTPase activity, on the other hand, was proportional to the KCl concentration (Table 1). Thus, filament bundling induced by Ca<sup>2+</sup> decreased the FtsZ nucleotide exchange rate, probably as a result of a decrease in the accessibility of the GTP binding site, but the exchange was still faster than hydrolysis, again showing that these two activities are not coupled.

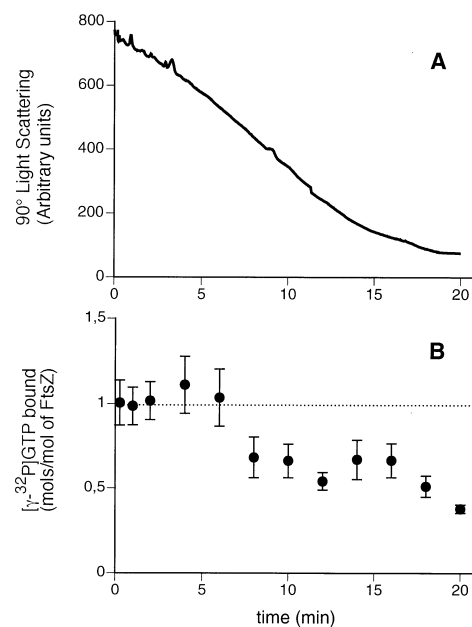
#### Molecular modelling of the FtsZ dimer

Our results suggest that the nucleotide binding site of FtsZ should remain relatively open to the medium even in the polymer subunits. To investigate this, a structure alignment was made between the three-dimensional structures of the tubulin dimer from porcine brain (Protein Data Bank entries 1TUB-A and 1TUB-B, corresponding to  $\alpha$ - and  $\beta$ -tubulin respectively) and FtsZ from *Methanococcus jannaschii*

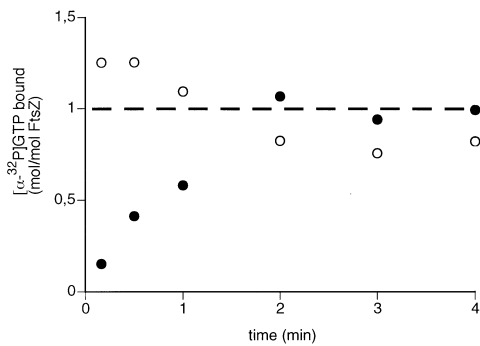
(Protein Data Bank entry 1FSZ). Each monomer of the tubulin dimer was substituted by a molecule of FtsZ according to the optimal pairwise alignment of the three-dimensional co-ordinates of each protein structure using an automated protein structure alignment algorithm (DALI; Holm and Sander, 1996). The resulting model for the FtsZ homodimer is almost equivalent to the model suggested by Löwe and Amos (1999) for the FtsZ protofilament structure. Comparison of the actual tubulin dimer (Fig. 5B) with the FtsZ homodimer model resulting from the alignment (Fig. 5A) shows some interesting differences related to the nucleotide binding site. Mainly, the interacting surface between  $\alpha$ - and  $\beta$ -tubulin results in a closed structure that immobilizes the GTP into the binding site. In contrast, in the model of the FtsZ homodimer, the interaction surfaces do not completely close the binding site, but leave an opening that might allow the exchange of the nucleotide even when in the FtsZ polymers.

#### Discussion

*In vitro*, FtsZ has GTP-binding and hydrolytic activities, and it is able to polymerize and assemble into a variety of structures depending on the assay conditions (Lu and Erickson, 1998). During cell division, FtsZ assembles into a ring at the division site. It is often assumed that assembly



**Fig. 3.** Nucleotide contents of FtsZ during polymerization. FtsZ (10  $\mu$ M) was incubated at 25°C in 50 mM Tris, pH 7.5, 100 mM KCl, 0.1 mM EDTA and 5 mM MgCl<sub>2</sub>, and buffered GTP was added to a concentration of 2 mM. A. Polymerization was followed by 90° light scattering. B. The GTP contents of the protein during the course of the reaction were analysed in parallel samples with a nitrocellulose filter-binding assay using [ $\gamma$ -<sup>32</sup>P]-GTP (0.25 Ci mmol<sup>-1</sup>). Each point is the mean  $\pm$  SEM of three independent experiments.



**Fig. 4.** Nucleotide exchange during FtsZ polymerization. FtsZ (10  $\mu$ M) polymerization was induced by preincubation for 1 min with 0.5 mM unlabelled GTP at 25°C in 50 mM Tris, pH 7.5, 100 mM KCl, 0.1 mM EDTA and 5 mM  $MgCl_2$  (open circles) or the same plus 10 mM  $CaCl_2$  (closed circles). At time 0 [ $\alpha$ - $^{32}P$ ]-GTP was added, and the incorporation of labelled GTP to FtsZ was measured using a nitrocellulose filter-binding assay. The dashed line is included as a reference and indicates 1 mol of nucleotide bound  $mol^{-1}$  FtsZ.

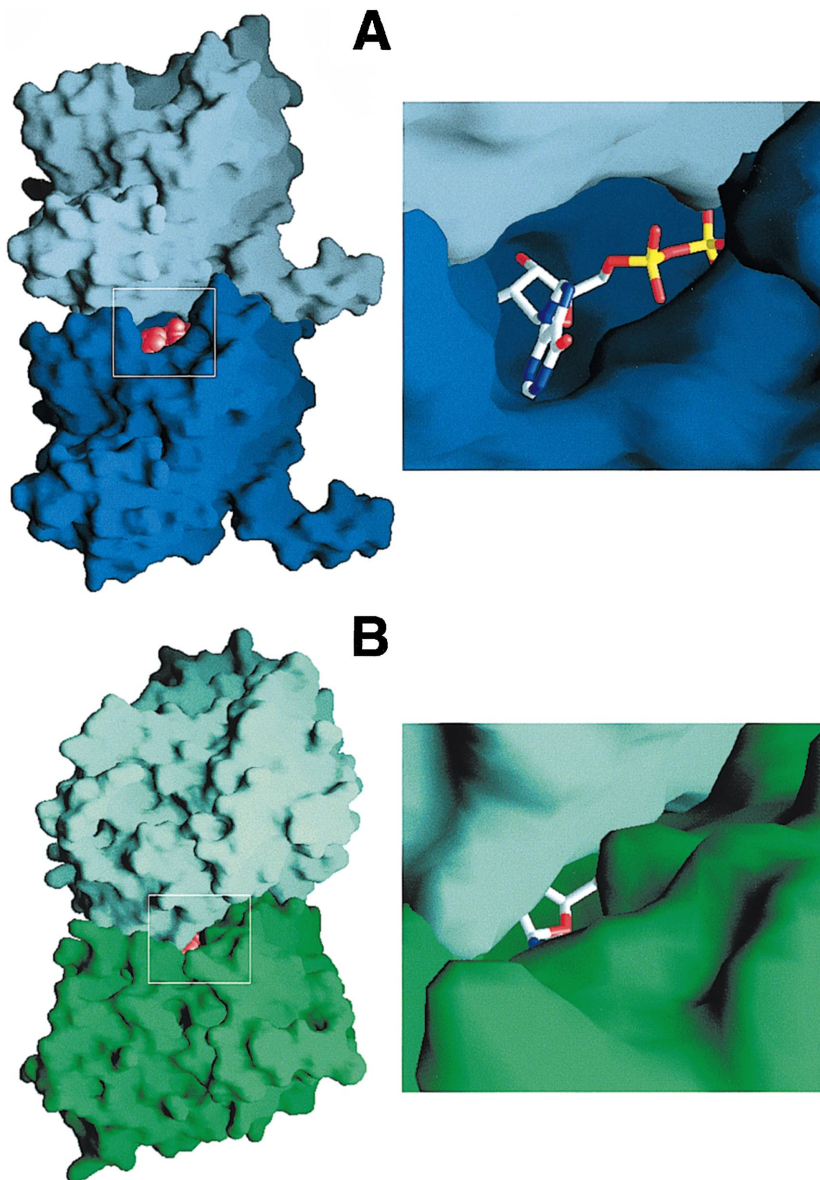
results from polymerization; however, the mechanisms that direct this process, as well as the physical nature of the structures formed *in vivo*, are not known. Several models have been proposed to describe the role that FtsZ might play in cell constriction, involving mechanisms that are either related to those of eukaryotic tubulins (Bramhill, 1997) or unrelated ones (Erickson, 1997). A critical difference between the models is the mode of nucleotide exchange, whether it occurs only in free monomers or also in the polymer subunits, because this would determine the role of the nucleotide in polymer assembly and disassembly (Desai and Mitchison, 1998). We find that, *in vitro*, FtsZ mostly contains GTP when it is in both non-polymerizing conditions (without GTPase activity) and polymerizing conditions (active GTPase). Our results cannot rule out completely that some fraction of the nucleotide bound in the polymers might be in the form of (GDP + Pi), but this is structurally equivalent to GTP. Although the presence of a small fraction of bound GDP should not be ruled out, it is clear that, during the first few minutes, when polymerization is maximal, GDP is not a significant fraction of the bound nucleotide. Moreover, FtsZ is able to exchange the bound guanine nucleotides very fast, the exchange rates being identical or very similar in polymerizing and non-polymerizing FtsZ. We conclude then that the exchange can occur in the free monomers and also in the polymer subunits. As the exchange is not coupled to the GTPase activity, we also conclude that both GTP and GDP can be exchanged in the subunits of the polymer (Fig. 6).

These data suggest that polymerization does not close the GTP binding site, and that the accessibility of this site is the same in the polymer subunits as in the free monomers. Molecular modelling of the structure of the FtsZ dimer showed the presence of an opening between the two subunits that seems to be large enough to allow

the movement of GTP and GDP through it. This suggests that the nucleotide binding site might be open even in the polymers, in agreement with our results. The nucleotide contained in the binding sites should then be in equilibrium with the free nucleotide pool and, consequently, nucleotide turnover cannot power polymer turnover (Desai and Mitchison, 1998). A role of GTP binding might then be to induce polymerization of FtsZ, as it strongly increases the affinity of the FtsZ–FtsZ interaction (Romberg *et al.*, 2001). The isodesmic polymerization model proposed by Romberg *et al.* (2001) includes a dissociation step after GTP hydrolysis, followed by GDP–GTP exchange. However, such a model is valid only for single-stranded protofilaments. As shown in Fig. 1, under physiologically relevant conditions (pH 7.5, 10  $\mu$ M FtsZ and in the presence of GTP,  $Mg^{2+}$  and  $K^+$ ), FtsZ protofilaments tend to form ribbons by longitudinal pairing. Stabilization by lateral interactions might prevent the dissociation of the GDP-containing subunits (as in tubulin) at least until the fraction of GDP-containing subunits is high enough to cause a sufficient number of nicks (or gaps) that would shred the multistranded ribbon. A plausible role for the rapid nucleotide turnover might be to maintain FtsZ in the GTP-bound form and, therefore, to maintain the polymerization state even when the GTPase is active (Fig. 6).

If assembly of the FtsZ rings requires the continuous presence of polymers, endowed with a GTPase activity and maintained by a stream of fresh GTP, it would be an energetically costly process, and only actively growing cells would be able to support it.

GDP-containing FtsZ polymers have been obtained by hydrolysis of GTP in the presence of  $Ca^{2+}$  and GTP- $\gamma$ -S (Scheffers *et al.*, 2000), and also by incubation of FtsZ with GDP in the presence of cationic lipids or DEAE-dextran (Erickson *et al.*, 1996; Lu *et al.*, 2000). It is known that  $Ca^{2+}$  induces the formation of FtsZ filament bundles (Yu and Margolin, 1997), and this suggests that the lateral interactions induced by  $Ca^{2+}$  between FtsZ protofilaments and the interactions of FtsZ filaments with charged surfaces or polymers might be similar and may stabilize the polymers even in the GDP-bound state. It has also been shown that, *in vitro*,  $Ca^{2+}$  inhibits the GTPase activity of FtsZ (Mukherjee and Lutkenhaus, 1999), and we report here that it also decreases the nucleotide exchange rate. In all these cases, the  $Ca^{2+}$  concentrations used were high (5–20 mM) compared with the levels of free  $Ca^{2+}$  in *E. coli* (200–300 nM; Holland *et al.*, 1999) and, therefore, it is doubtful whether those results can be extrapolated to describe the behaviour of FtsZ in the living cell. Nevertheless, the lateral interactions between FtsZ protofilaments found in the presence of  $Ca^{2+}$  may not be an artifact, but may mimic some of the interactions established in the division septum. GTP-containing FtsZ



**Fig. 5.** A model for the homodimerization of FtsZ. The structure of *Methanococcus jannaschii* FtsZ (PDB entry 1FSZ) was aligned with those of the  $\alpha$ - and  $\beta$ -tubulin subunits (PDB entries 1TUB-A and 1TUB-B) using the DALI algorithm, and a model of the FtsZ dimer was made by substituting the tubulin monomers for the aligned FtsZ structures.

A. Solvent-accessible surface representation of the model for FtsZ dimerization. The surface was calculated using GRASP. A molecule of GDP (extracted from the same PDB entry 1FSZ) is shown located between the two structures (red spheres, left; coloured sticks, right).

B. Solvent-accessible surface of the tubulin dimer calculated with GRASP. A molecule of GTP (from PDB entry 1TUB; red spheres, left; coloured sticks, right) is trapped between the two subunits.

could form straight filaments, which might bind to the membrane, either directly or through anchoring membrane proteins, such as ZipA (Hale and de Boer, 1997; RayChaudhuri, 1999). Association to the membrane could also stabilize the filaments even after hydrolysis of the nucleotide.

In summary, we can envisage at least three stages in the action of FtsZ in cell division. First, an isodesmic polymerization of FtsZ to form single-stranded protofilaments (Rivas *et al.*, 2000; Romberg *et al.*, 2001); this would require a continuous supply of GTP. Next, the assembly of protofilaments into a higher order structure would involve a switch to co-operative interactions (and/or heteromolecular interactions). This stage would include protofilament pairing and formation of bundles. These structures might convert the energy of GTP hydrolysis into

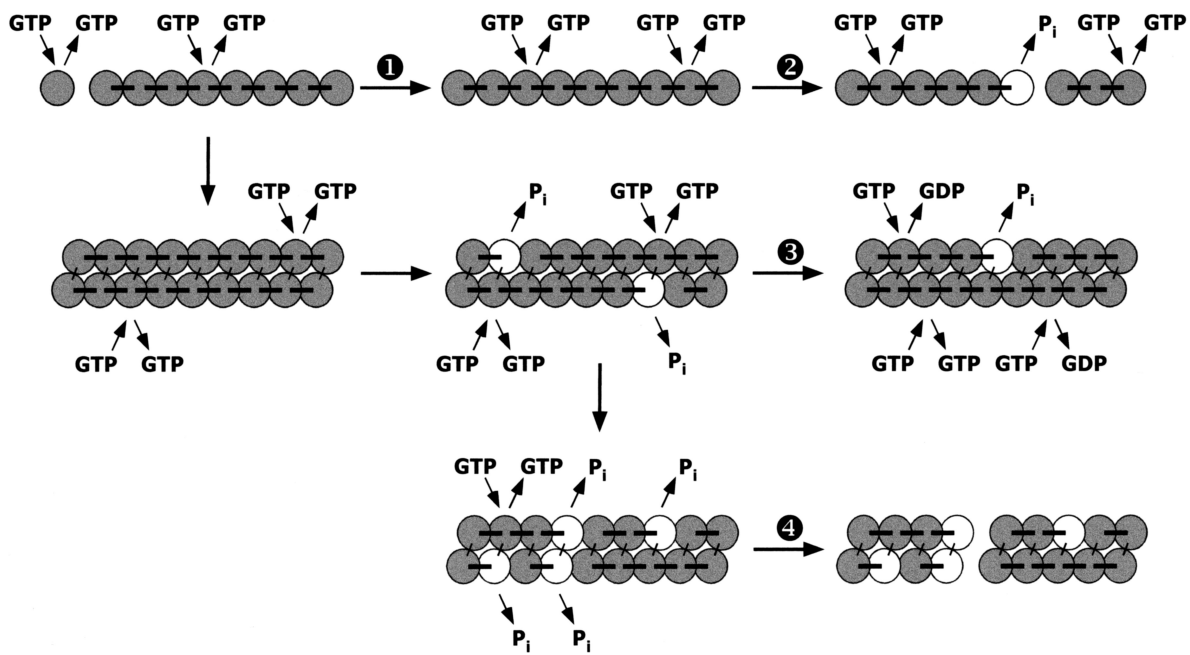
mechanical forces (Díaz *et al.*, 2001). Finally, reorganization of the assembled FtsZ would occur when a sufficient number of GDP-containing subunits produce shredding (Fig. 6; Romberg *et al.*, 2001). In principle, the whole process of septation may involve several of these cycles to effect the closure of the cell wall.

## Experimental procedures

### Chemicals

CaCl<sub>2</sub>, MgCl<sub>2</sub>, EDTA, Tris, PIPES and GTP were all from Sigma. Nitrocellulose filters were cut from Protran<sup>®</sup> membrane (Schleicher and Schuell). [ $\alpha$ -<sup>32</sup>P]-GTP (3000 Ci mmol<sup>-1</sup>) and [ $\gamma$ -<sup>32</sup>P]-GTP (5000 Ci mmol<sup>-1</sup>) were from Amersham Pharmacia Biotech. Polygram CEL 300 PEI/UV<sub>254</sub> cellulose plates





**Fig. 6.** A model for FtsZ polymerization *in vitro*. At pH 6.5 and low protein concentration (Romberg *et al.*, 2001), FtsZ monomers containing GTP at the nucleotide binding site (grey circles) polymerize according to an isodesmic model (1). Hydrolysis of the nucleotide leaves GDP at the binding site (white circles) and is followed by rapid dissociation at the GDP-FtsZ–GTP-FtsZ interface (2). Under more physiological conditions, FtsZ polymers tend to group, forming ribbons composed of two or more protofilaments. Hydrolysis of GTP produces nicks, but the fast nucleotide exchange along the polymer quickly replaces GDP with GTP, restoring the interaction with the neighbour monomers (3). Upon exhaustion of GTP, the fraction of GDP-bound monomers increases, and FtsZ depolymerizes (4). The thick horizontal lines between subunits represent the primary interaction between GTP-containing monomers, and the thin oblique lines represent the lateral interactions between protofilaments.

were from Macherey-Nagel. Other reagents were analytical grade from Merck or Sigma, except when indicated otherwise.

#### FtsZ purification

FtsZ was purified by the  $\text{Ca}^{2+}$ -induced precipitation method as described by Rivas *et al.* (2000). The protein was stored frozen at  $-80^{\circ}\text{C}$ . Before use, the storage buffer was changed to polymerization buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 100 mM KCl) using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech) coupled to an Amersham Pharmacia Biotech fast protein liquid chromatography (FPLC) system.

#### Light scattering analysis of FtsZ assembly

Analysis of FtsZ assembly by  $90^{\circ}$  light scattering was performed as described by Mukherjee and Lutkenhaus (1999). Light scattering was measured in a Hitachi F-2500 fluorescence spectrophotometer. Excitation and emission wavelengths were set to 350 nm, with slit widths of 5 nm. All reactions were performed at  $25^{\circ}\text{C}$ . FtsZ was incubated in polymerization buffer in a fluorimeter cuvette. After 5 min of equilibration, GTP was added, and the reactions were monitored for at least 15 min.

#### Electron microscopy

FtsZ ( $10\ \mu\text{M}$ ) was incubated in 100  $\mu\text{l}$  of polymerization buffer.

GTP was added to 0.5 mM and, after 1 min, the samples were applied to 400-mesh collodion-coated glow-discharged grids, negatively stained with 2% uranyl acetate, and inspected with a Jeol 1200EXII electron microscope.

#### GTP binding

GTP binding was monitored by a nitrocellulose filter-binding assay. Reaction mixtures (10–20  $\mu\text{l}$ ) were vacuum filtered through nitrocellulose, and the filters were washed with 2 ml of ice-cold wash buffer [20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM  $\text{MgSO}_4$ , 0.005% Triton X-100 and 20 mM  $(\text{NH}_4)_2\text{SO}_4$ ]. The filters were dried, and the amount of radioactivity was determined by Cerenkov counting in a scintillation counter. To determine the specific activities of the reaction mixtures (c.p.m.  $\text{pmol}^{-1}$  nucleotide), small aliquots were diluted and spotted on dry nitrocellulose filters, and these were counted together with the sample filters. Background binding of radioactive nucleotides to the filters, measured in the absence of protein, was very low and was subtracted. Binding of  $^{32}\text{P}$  resulting from hydrolysis of  $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$  was very low, reaching background levels when hydrolysis was complete; including 5 mM  $\text{P}_i$  in the reactions had little effect on the measurement of  $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$  binding. In the presence of  $\text{Ca}^{2+}$ , on the other hand, the signal increased with time, eventually becoming very high, presumably as a result of the formation of calcium phosphate complexes that were retained by the filters; this prevented the use of  $[\gamma\text{-}^{32}\text{P}]\text{-GTP}$  in the presence of  $\text{Ca}^{2+}$ . Nucleotide contents are presented as mean  $\pm$  (SEM) of at least three independent measurements.

In time course experiments, single points were taken for each time. The protein concentration (typically 10  $\mu\text{M}$ ) was always well below the nominal capacity of the membranes given by the manufacturer (80  $\mu\text{g cm}^{-2}$ ). However, the protein concentration in the eluate was measured in control experiments, which showed that all the protein was indeed retained by the filters. Therefore, the amount of protein bound to the filters was taken to be the input protein.

The kinetic experiments were analysed according to an association binding model (Motulsky and Neubig, 2000). The rates were calculated by non-linear regression to a one-phase exponential association model.

In some cases, the binding was too fast to calculate any meaningful rate. In these cases, a semi-quantitative (minimal) estimate was obtained from the relation  $k_{\text{ob}} = 0.693/t_{1/2}$ , where  $t_{1/2}$  is the time to reach half-maximal binding, and assuming this time to be lower than 10 s, which is the time of the first data point; this estimate is therefore very conservative and gives a lower limit for the actual exchange rate.

#### GTPase activity

The GTPase activity of FtsZ was analysed by thin-layer chromatography. The reactions were stopped by mixing 2  $\mu\text{l}$  of the samples with 2  $\mu\text{l}$  of 50 mM EDTA, pH 8.0, and stored on ice. From these mixtures, 2  $\mu\text{l}$  was spotted on PEI-cellulose plates and developed in 0.5 M LiCl–1 M formic acid. The plates were dried and analysed with a phosphorimager (Personal Molecular Imager FX; Molecular Dynamics). GTPase turnover rates were calculated from the linear part of the GDP accumulation curves.

#### Molecular modelling

The three-dimensional structures of the tubulin dimer from porcine brain (Nogales *et al.*, 1998) and FtsZ protein from *Methanococcus jannaschii* (Löwe and Amos, 1998) were obtained from the Protein Data Bank (entries 1TUB and 1FSZ respectively). The structure alignment was performed using DALI (Holm and Sander, 1996; <http://www2.ebi.ac.uk/dali>). The surface representation of the modelled proteins was performed using GRASP (Nicholls *et al.*, 1993).

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