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Independence between GTPase active sites in the *Escherichia coli* cell division protein FtsZ

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ABSTRACT

We have analyzed the substrate kinetics of the GTPase activity of FtsZ and the effects of two different GTPase inhibitors, GDP and the slowly hydrolyzable GTP analogue GMPCPP. In the absence of inhibitors the GTPase activity follows simple Michaelis–Menten kinetics, and both GDP and GMPCPP inhibited the activity in a competitive manner. These results indicate that the GTPase active sites in FtsZ filaments are independent of each other, a feature relevant to elucidate the role of GTP hydrolysis in FtsZ function and cell division.

Structured summary of protein interactions:
FtsZ and **FtsZ** bind by light scattering (View interaction).

1. Introduction

FtsZ is a GTPase involved in division in bacteria, euryarchaea and chloroplasts [1,2]. In vitro FtsZ assembles in a GTP-dependent manner forming polymorphic polymers whose structures depend on the reaction conditions. In solution the main polymers are thought to be cyclic single stranded filaments about one hundred subunits long [3], while when bound to a surface a variety of structures of different shapes and sizes are seen by electron microscopy and atomic force microscopy [4,5]. In spite of much work by several groups, the dynamics of the filaments and their biological role remain controversial [1,2,6].

In the presence of Mg^{++} and GDP purified FtsZ assembles through an isodesmic mechanism forming short single-stranded oligomers [7]. In the absence of cations it is monomeric, binds GTP [8], but does not have enzymatic activity. Addition of Mg^{++} and K^{+} ions promotes FtsZ–GTP polymerization, and formation of a GTP-binding site in the interface between every two subunits [7–9].

The GTPase activity of FtsZ has been measured previously [10,11]. At neutral pH and in the presence of saturating concentrations of GTP, Mg^{++} and K^{+} the hydrolysis rate is in the order of 5 mol GTP/mol FtsZ/min [10–12], and the activity follows Michaelis–Menten kinetics with a K_m value of ~80 μM [10]. This value is much larger than the nucleotide binding affinities which are on the nanomolar range for both GTP and GDP [13,14], consequently the polymers contain a substantial amount of GTP [12,15,16]. These data suggest that some steps of the GTPase activity, different from nucleotide binding, are rate-limiting in FtsZ polymer dynamics [15].

There are currently several models on FtsZ filament assembly and dynamics and their role in cell constriction [1,2], but there are several details still lacking, such as the location of active sites within filaments, the number of active sites per filament, or how nucleotide hydrolysis relates to protein subunit dynamics.

To gain insight into these aspects of the catalytic activity of FtsZ we have analyzed the enzyme kinetics of *Escherichia coli* FtsZ with GTP and with the slowly-hydrolyzable analogue GMPCPP [17,18].

2. Materials and methods

2.1. Reagents

Guanine nucleotides GDP and GTP were from Sigma–Aldrich. The nucleotide analogue GMPCPP was purchased from Jena Bioscience GmbH (Germany). Other analytical grade chemicals were from Merck or Sigma–Aldrich.

2.2. Protein purification

E. coli FtsZ was purified by the calcium-induced precipitation method as described [7]. To measure the GTPase activity at low

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GTP concentration (below 0.1 mM), the protein was desalted in a 5 ml Hi-Trap Desalting column equilibrated in 50 mM Tris pH 7.5, 250 mM KCl, without Mg^{++}. Immediately after elution GTP was added to the fraction containing the protein to a concentration of 50 μM.

2.3. GTPase activity

The GTPase activity was measured by the malachite green method [19]. Reactions were done at 22 °C in 100 μl of 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 250 mM KCl, with 10 μM FtsZ. Ten microliter fractions were taken at different times and mixed with 40 μl of the same buffer containing EDTA (65 mM) to stop the reaction. The green malachite-molybdate reagent was added and absorbance at 620 nm was measured. A phosphate standard curve was done with Na_2HPO_4. Activities were determined from the slope of the linear part of the phosphate accumulation curve. The standard errors calculated for these measurements were always on the order of 5% of the measured value. All the experiments were repeated two to four times. Error bars in the figures represent the standard deviations of independent replicates performed with the same protein batch.

The GTPase activity was analyzed by thin-layer chromatography and [α-^{32}P]-GTP (3000 Ci/mmol, Perkin-Elmer) as described [12]. Kinetic parameters were calculated using GraphPad Prism 5.0.

2.4. FtsZ polymerization

Polymerization was analyzed by 90° light scattering in a Hitachi F-2500 fluorescence spectrophotometer as described [12]. Reactions were done at 25 °C in 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 250 mM KCl with 0.5 mM GTP and/or 0.5 mM GMPCPP.

3. Results

3.1. Kinetics of the GTPase activity of FtsZ

The GTPase activity of FtsZ has been found to follow Michaelis–Menten kinetics [10]. However, measurements done at low GTP concentrations might have been flawed by the GDP contents of the purified protein. To circumvent this problem we exchanged the nucleotide in purified protein preparations by passage through a desalting column equilibrated in buffer without Mg^{++} and addition of GTP to a concentration of 50 μM after elution. Under these conditions the protein binds GTP, but cannot hydrolyze it [8]. The GTP concentration was then adjusted to the desired value upon dilution of the protein into the reaction mix, and the reaction was triggered by adding Mg^{++} to a concentration of 5 mM. Our protein preparations did not show time lags in catalytic activity, so initial GTPase rates could be calculated from phosphate production curves. The activity approached hyperbolic kinetics that could be fitted to a Michaelis–Menten curve with V_{max} = 6.0 ± 0.4 mols GTP/mol FtsZ/min and K_m = 0.3 ± 0.05 mM (Fig. 1), with a slight deviation at the lower GTP concentrations (Fig. 1, inset).

3.2. Competitive inhibition of the FtsZ GTPase activity by GDP

GDP competes with GTP for binding to the active sites [20], and an excess of GDP inhibits polymerization and induces depolymerization [8,20]. To study the mechanism of inhibition, the GTPase activity was measured over a range of GTP concentrations in the presence of 0.1 mM GDP, 0.5 mM GDP and 1 mM GDP. The Lineweaver–Burk plot indicated a competitive inhibition mechanism (Fig. 2A), and non-linear fitting of the whole dataset to a competitive inhibition model yielded a K_i of 0.47 ± 0.09 mM GDP (Table 1).

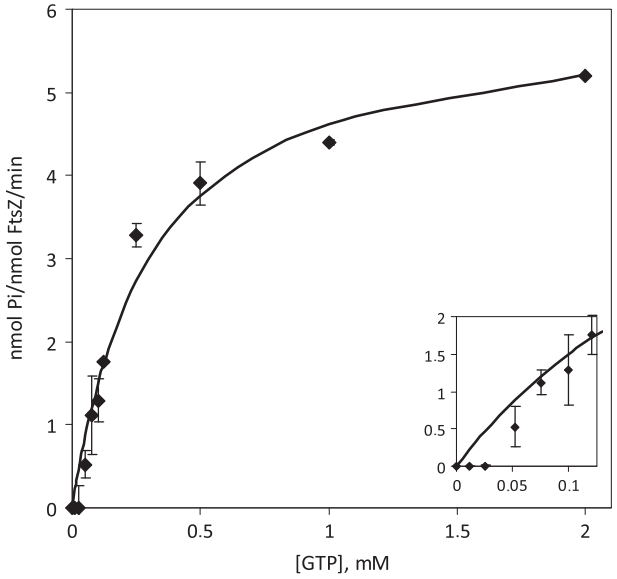

Fig. 1. GTPase kinetics of FtsZ. Initial rates were obtained from the slopes of phosphate accumulation curves and fitted to a Michaelis–Menten model with a V_{max} = 6.0 ± 0.4 nmols GTP/nmol FtsZ/min and a K_m = 0.3 ± 0.05 mM (Table 1). Data points are average and standard deviation from three measurements. Inset: expanded view of the plot at low GTP concentrations.

3.3. Hydrolysis of GMPCPP

The nucleotide analogue guanosine 5′-α-β-methylene triphosphate (GMPCPP) is known to be slowly hydrolyzed [17,18]. To measure GMPCPP hydrolysis the reaction was followed for 60 min, a period much longer than that used for GTP. At 0.5 mM GMPCPP the hydrolysis rate was 0.18 mols GMPCPP/mol FtsZ/min, 20-fold lower than the GTPase rate measured at the same nucleotide concentration (3.9 ± 0.91 mols GTP/mol FtsZ/min). No accumulation of phosphate could be detected during the first two minutes of the reaction, which is the period used to measure the GTPase activity. Hence, hydrolysis of GMPCPP will not interfere with the measurement of GTP hydrolysis in assays with the two substrates, and thus the effect of GMPCPP on the GTPase activity might be measured.

3.4. Competitive inhibition of the FtsZ GTPase activity by GMPCPP

Addition of GMPCPP to the reaction mixtures decreased the rate of GTP hydrolysis. To make certain that the Pi detected proceeded only from GTP, and not from an increase in the hydrolysis of GMPCPP, the experiment was repeated in the same conditions but using [α-^{32}P]-GTP to detect the radiolabeled phosphate by thin-layer chromatography. The GTPase activities measured in the presence and absence of GMPCPP were the same with the radioisotopic and the colorimetric assays, confirming that the Pi detected with the colorimetric assay proceeded from the hydrolysis of GTP, and not from GMPCPP.

We next studied the effects of the two nucleotides in polymerization by light scattering and electron microscopy. Upon addition of GTP, light scattering shows a rapid increase due to polymer formation, followed by a slow decrease parallel to GTP depletion and GDP buildup. Addition of GMPCPP triggers the same initial increase, but this is followed by a second phase of slow increase, likely due to filament aggregation (Fig. 3). Mixtures of the two nucleotides show the same behavior as GTP alone (Fig. 3). Electron microscopy showed networks of single and multiple strand filaments and bundles, with some single strand circular filaments

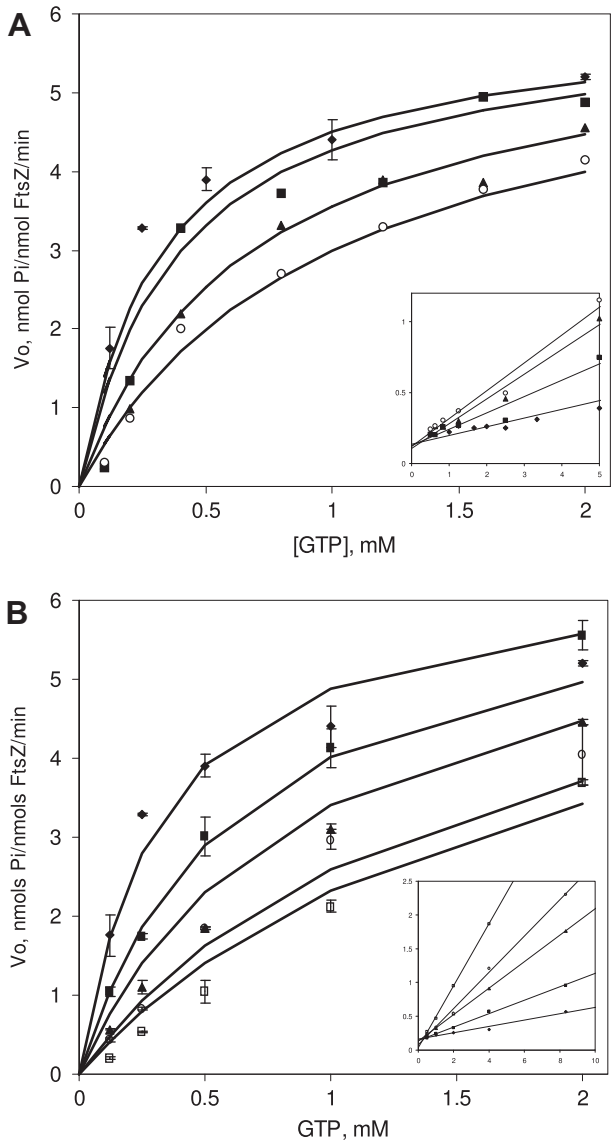

Fig. 2. GTPase kinetics of FtsZ measured in the presence of (A) 0 mM (♦), 0.1 mM (■), 0.5 mM (▲) and 1 mM GDP (○), and (B) 0 mM (♦), 0.1 mM (■), 0.2 mM (▲), 0.4 mM (○) and 0.5 mM GMPCPP (□). Data points are average and standard deviation from two (GMPCPP) or three (GTP) measurements. Inset: Lineweaver–Burk plots.

Table 1
Kinetic parameters of FtsZ GTPase activity. The table shows the values estimated by non-linear fitting of the data to Michaelis–Menten and competitive inhibition models. In parenthesis: 95% confidence intervals.

Inhibitor	K_m (mM)	K_i (mM)	V_{max} (mol GTP/mol FtsZ/min)
None	0.30 (0.17–0.42)	–	6.0 (5.1–6.9)
GDP	0.33 (0.22–0.45)	0.47 (0.27–0.66)	5.9 (5.3–6.7)
GMPCPP	0.33 (0.24–0.41)	0.11 (0.08–0.14)	6.5 (6.0–6.9)

scattered throughout the preparations, but there were not significant differences among GTP, GMPCPP or mixtures of GTP and GMPCPP.

Next, the GTPase activity was measured over a range of GTP concentrations in the presence of increasing amounts of GMPCPP. Similarly to GDP inhibition, the Lineweaver–Burk plot indicated a competitive inhibition mechanism (Fig. 2B), and non-linear fitting

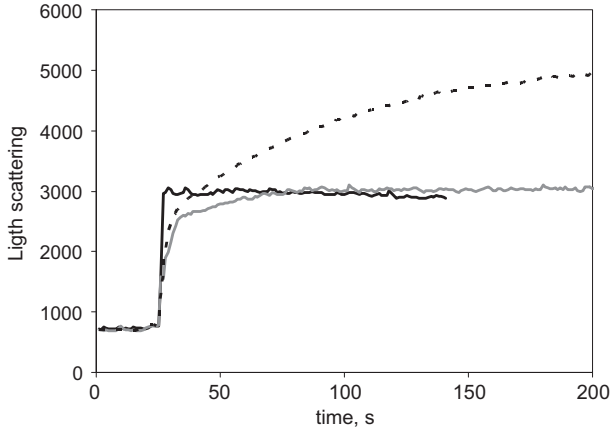

Fig. 3. Polymerization of FtsZ in the presence of 0.5 mM GTP (black line), 0.5 mM GMPCPP (dotted line) and a mixture of GTP and GMPCPP, 0.5 mM each (gray line).

to a competitive inhibition model yielded a K_i of 0.11 ± 0.01 mM GMPCPP (Table 1).

4. Discussion

In spite of the heterogeneity in polymer size and shape seen under the electron microscope [4], in solution FtsZ–GTP polymers have a monodisperse size distribution [3]. This indicates that the polymers have a well-defined and homogeneous size, enabling the interpretation of biochemical data. A polymeric structure suggests that the activity of one binding site might be affected by that of nearby sites giving rise to complex enzyme kinetics. Quite the opposite, we have found that the GTPase activity follows Michaelis–Menten kinetics, confirming previous results [10]. The slight deviation seen at the lower GTP concentrations is likely methodological because hydrolysis at low GTP concentrations has been detected previously with other methods [15]. The kinetic behavior of FtsZ suggests that the active sites within filaments are independent. Furthermore, we have studied the kinetics of two different inhibitors of the GTPase activity. GDP was already known to competitively inhibit the activity, probably by sequestering monomers from the pool available for polymerization [20]. GMPCPP on the contrary is a substrate of FtsZ that is able to induce polymerization but is slowly hydrolyzed. This suggested that GMPCPP might inhibit the GTPase activity in FtsZ filaments by occupying active sites within polymers, not by sequestering monomers away from the polymeric fraction. The fact that it does inhibit competitively reinforces the idea that GTPase active sites of FtsZ are independent of each other. If there were some kind of interaction between active sites in FtsZ polymers it would be expected that inhibition by GMPCPP should be non-competitive because every site occupied by GMPCPP would affect the activity of more than one active site.

So, the GTPase activity of FtsZ behaves much like it would be expected for a monomeric enzyme.

If all the GTP-bound sites within FtsZ filaments are non-interacting, catalytically active sites (Fig. 4A), then the different subunits within a filament should hydrolyze GTP in a random manner, and then either GDP might be exchanged within the filaments [12] or the filaments might be fragmented at the interfaces that contain GDP. Alternatively, it might be thought that every filament has a single GTPase active site and the whole filament behaves as a monomer. The active sites might be one or the two extremes of the filaments (Fig. 4B), or it might be an internal site separating the filament into a GTP-bound part and a GDP-bound part (Fig. 4C). In this case, the filament dynamics might be driven by treadmilling or dynamic instability mechanisms, there would be an interaction

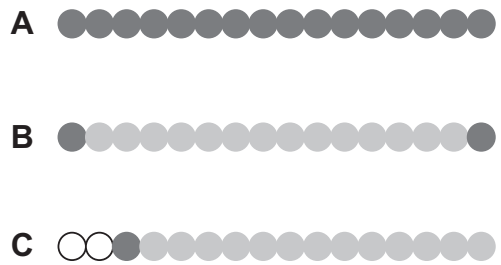

Fig. 4. Schematic view of three models of FtsZ filaments with independent GTPase active sites. (A) All the GTP bound subunits are active (dark gray circles), hydrolysis occurs randomly and is followed by nucleotide exchange in the filament or by filament fragmentation. (B) All the subunits are bound to GTP, only the terminal sites are active (dark gray circles), the central subunits (light gray circles) are not active. This model was suggested by Martín-García, Gómez-Puertas et al. based on molecular dynamics simulation of FtsZ filaments. (C) Only one subunit per filament is active (dark gray circle); *upstream* subunits are bound to GTP (light gray circles) and *downstream* subunits are bound to GDP (white circles).

between the active site and the next site, but each filament would have a single active site that would not interact with the active sites of other filaments.

These different models are related to different roles of the GTPase activity in FtsZ function. In the all-sites active model the function of the GTPase might be related to force generation by nucleotide-dependent filament bending [1], while in the models of single or few active sites it might more related to the regulation of filament length and dynamics, in which case cell constriction should be driven by lateral interactions between filaments [21,22], or by nucleotide-independent filament bending mechanisms [23,24].

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