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Interaction between *Bacillus subtilis* YsxC and ribosomes (or rRNAs)

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ABSTRACT

YsxC is an essential P-loop GTPase, that binds to the 50S ribosomal subunit, and is required for the proper assembly of the ribosome. The aim of this study was to characterize YsxC ribosome interactions.

The stoichiometry of YsxC ribosome subunit complex was evaluated. We showed that YsxC binding to the 50S ribosomal subunit is not affected by GTP, but in the presence of GDP the stoichiometry of YsxC-ribosome is decreased. YsxC GTPase activity was stimulated upon 50S ribosomal subunit binding. In addition, it is shown for the first time that YsxC binds both 16S and 23S ribosomal RNAs.

1. Introduction

Ribosomes are cellular organelles composed of approximately 60% ribosomal RNA (rRNA) and 40% protein that catalyze protein synthesis in the cell. The bacterial 70S ribosome contains about 55 ribosomal proteins (depending on the species) and 3 ribosomal RNAs, organized into two subunits: the small (30S) and large (50S) ribosomal subunits. Formation of the ribosomal particle involves a complex series of processes, *i.e.*, synthesis, processing and modification of both rRNA [1,2] and ribosomal proteins [3,4], and assembly of the components. Recently, by using quantitative mass spectrometry, Chen and Williamson [5] were able to provide a clear picture of in vivo 30S and 50S assembly. A variety of non-ribosomal factors are involved in the ribosome biogenesis process. Among them, several phosphate-binding loop (P-loop) GTPases (YlqF/RbgA [6,7], CgtAE/ObgE [8,9], YphC [10] and YsxC [10,11]) have been suggested to be necessary for bacterial ribosome assembly because dissociated ribosomal subunits accumulate in cells depleted in these GTPases.

YsxC is an essential P-loop GTPase in in *Escherichia coli* [12,13], *Bacillus subtilis* [14] and *Staphylococcus aureus* [11]. The protein from *B. subtilis* [15,16] was crystallized as a single domain protein of 22kDa. The protein undergoes conformational changes during nucleotide binding [16]. YsxC associates primarily with the 50S subunit of the ribosome [11,17] and, when expressed in *E. coli*, co-purifies with the ribosomal fraction [17]. In this study, we thoroughly characterized YsxC association with the ribosome, by examining the role of nucleotides, quantifying the number of YsxC molecules bound to the ribosomal subunits, and investigating YsxC binding to the ribosomal RNAs.

2. Materials and methods

2.1. Preparation of recombinant and wild-type YsxC proteins

Recombinant *B. subtilis* $(His)_6$YsxC was cloned, expressed and purified to homogeneity, as described [17], and stored in 50 mM NaPO4 buffer, pH 8, containing 15% (v/v) glycerol and either 0.75 M NaCl or 0.5 M KCl.

Mutants in the P-loop motif were generated by the Quickchange protocol (Stratagene) with the pET15b-*ysxC* [17] used as a template, and the oligonucleotides indicated (Supplementary Table 1). The constructs were confirmed by DNA sequencing (Genome express). Standard genetic techniques were used [18]. Expressions were done at 18 °C. Purifications of the mutants were the same as for the wild-type protein.

Abbreviations: P-loop, phosphate-binding loop; GMPPNP, guanylyl-imidodiphosphate; rRNA, ribosomal RNA; Tm, melting temperature; TSA, thermal shift assay; DTT, dithiothreitol

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2.2. Assay of GTPase activity

GTPase activity was followed by spectrophotometric recording of NADH oxidation in the presence of a GTP-regenerating system, at 37 °C. Enzyme (20 μg) was added to the assay medium (50 mM $NaPO_4$, pH 8.0, 0.15 M NaCl, 30 mM KCl, 4 mM phosphoenolpyruvate, 0.4 mM NADH, 8 μg of pyruvate kinase, 4 μg of lactate dehydrogenase, 2 mM $MgCl_2$, and 1 mM GTP, 200 μl final volume), as detailed [19]. GTP was regenerated from GDP by pyruvate kinase, with a concomitant production of pyruvate from phosphoenolpyruvate. Pyruvate was then converted to lactate by lactate dehydrogenase, with, concomitantly, NADH oxidation to NAD^+, which was followed by the change in absorbance at 340 nm for 10 min ($\varepsilon NADH_{340nm}$ = 6220 (mol^{-1} cm^{-1}). The experiments were performed with a Safas UVmc^2 spectrophotometer. Control experiments were systematically performed in the absence of nucleotides (protein alone) or in the absence of proteins (nucleotides alone) and the rates of NADH disappearance in all cases were negligible. The activities were expressed as μmol GTP hydrolyzed/min/mg protein.

2.3. Ribosome purification and preparation of 30S and 50S subunits

Ribosomes from *B. subtilis* (strain 168) were prepared following the detailed protocol of Fechter et al. [20] and separated on a 5–20% sucrose gradient into 30S and 50S subunits, as described [17]. 30S and 50S subunits fractions were stored at −80 °C (final concentration: 12 μM).

2.4. Binding of YsxC to ribosome

A filtration-based binding assay was used [21]. YsxC was incubated with 0.2 μM ribosome in 80 μl of 30 mM Tris–HCl, pH 7.5, 10 mM Mg acetate, 60 mM NH_4Cl, 60 mM KCl, 2.5 mM dithiothreitol (DTT) at 30 °C for 10 min. The mixture was applied onto Centricon YM-100 (Millipore) which was centrifuged for 5 min at 3000*g* to retain the ribosome-bound YsxC, and washed twice with 100 μl of the same buffer. 40 μl of buffer was applied onto the filter for 1 min and the ribosome-bound YsxC was collected from the inverted filter by centrifugation (3000*g* for 1 min). The recovered YsxC was detected by Western blotting using an India™ HisProbe-HRP (Pierce) and quantified. Control experiments without ribosomes were done.

2.5. In vitro synthesis of 5S, 16S, and 23S RNA

5S, 16S, and 23S DNAs were amplified from *B. subtilis* genomic DNA by PCR using the primers depicted in Supplementary Table 2. The cloning into the pET21 vector (Novagen) of the amplified products (116, 1470, and 2926 bp for 5S, 16S, and 23S DNA, respectively) were done according established methods [18]. All constructs were checked by sequencing.

In vitro transcription reactions were realized using the RiboMAX™ Large scale RNA Production Systems kit (Promega). DNA templates were linearized by *EcoRI* (5S DNA) or *XhoI*, (16S and 23S RNA), prior to in vitro transcription at 37 °C for 4 h. In vitro transcripts were further purified using the MEGAclear™ kit (Ambion). The quality of in vitro transcripts was examined by denaturing gel electrophoresis (1% agarose for 16S and 23S RNA) or 6% acrylamide gel electrophoresis for 5S RNA). RNA concentration was determined by absorbance at 260 nm.

2.6. 5′-end rRNA biotinylation

rRNAs were dephosphorylated by calf intestine phosphatase (CIP, 0.01 unit/pmole of 5′ end of RNA) at 37 °C for 1 h. CIP was removed by phenol extraction. After ethanol precipitation, RNAs were phosphorylated at 37 °C for 1 h by T4 polynucleotide kinase using UTP-biotin (10 units polynucleotide kinase/100 pmol RNA, 25 pmol Biotin-11-UTP (Fermentas)). Biotinylated 16S and 23S rRNAs were further purified using the MEGAclear™ kit (Ambion). 5S rRNA was purified by ammonium acetate precipitation.

2.7. rRNA blot overlay assay

rRNA blot overlay assays were performed following the protocol described by Palaniyandi et al. [22]. In brief, after YsxC migration on a 14% SDS–PAGE and electrotransfer to nitrocellulose, the membranes were washed three times 10 min and incubated in binding buffer (50 mM Tris–HCl, pH 8.0, 150 mM KCl, 0.5 mM DTT, 1 mM EDTA, 10 μg/ml *E. coli* tRNA) in the presence of biotinylated rRNA at 4 °C for 16 h. The blots were washed three times with binding buffer at room temperature, 5 min per wash. Biotin-labeled RNA was detected using the Biotin Chromogenic Detection Kit (Fermentas).

2.8. Thermal shift assay (TSA)

The assay used a real-time PCR machine (CFX Manager), the iCycler iQ Real-Time Detection System (Bio-Rad), which monitors fluorescence changes of sypro orange dye (excitation/emission: 490/575 nm) as it interacts with the protein undergoing thermal unfolding, in thin-walled 96-well PCR plates. Each well (25 μl) contained 2 μg protein and 2 μl of the fluorescent Sypro orange dye solution (Molecular Probes, 500× in DMSO, diluted 5 times in water), in 50 mM $NaPO_4$, pH8.0, 0.15 M NaCl and was heated from 20 to 100 °C in 0.2 °C steps.

3. Results and discussion

3.1. The stoichiometry of YsxC-ribosome

We had shown by sucrose density gradient that YsxC associated mainly with the free 50S ribosomal subunit in the absence of exogenous nucleotides [17]. However, the amount of bound YsxC had not been quantified previously. To better characterize YsxC-ribosome associations, the stoichiometry of the complex was evaluated, using a filtration-based binding assay [21].

The number of YsxC molecules bound per 70S, 50S or 30S particle was determined at different YsxC-particle ratios in the binding assay (up to 10-fold YsxC excess) in buffer 1 (Fig. 1a) (30 mM Tris–HCl, pH 7.5, 10 mM Mg acetate, 60 mM NH_4Cl, 60 mM KCl, 2.5 mM DTT). Control experiments without ribosomes were done to check that there was no non-specific interaction between YsxC and the filter membrane (Fig. 1a). The stoichiometry of the YsxC-50S complex was determined to be 1 and a high excess of YsxC did not significantly affect this ratio (see Table 1 and Ref. [23] for establishing the amount of recovered protein and ribosome). By contrast, there was no YsxC binding to 30S, and a faint binding to 70S ribosome (perhaps due to partial ribosome dissociation) (Fig. 1a).

We also investigated a buffer we used in a former study (buffer 2: 10 mM Tris–HCl, pH 7.4, 10 mM Mg acetate, 30 mM KCl, [17]), and the buffer used by Nakano et al. [21], (buffer 3: 10 mM Tris–HCl, pH 7.4, 8.2 mM Mg acetate, 50 mM NH_4Cl, 1 mM DTT, 0.3 mM EDTA), for measuring YlqF binding to the 50S subunit by the same filtering technique (Fig. 1b and 1c and Table 1). The ionic strength of buffer 2 was lower than that of buffer 1, resulting in more YsxC binding to all ribosomal particles (Table 1). YsxC had a strong tendency to stick to the ribosomal subunits in buffer 3: the more YsxC we added, the more binding we observed (Fig. 1c). As the ionic strengths of buffers 1 and 3 were comparable,