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The *C*-terminal α-helix of YsxC is essential for its binding to 50S ribosome and rRNAs

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ABSTRACT

YsxC is an essential P-loop GTPase that interacts with the 50S subunit of the ribosome. The putative implication in ribosome binding of two basic clusters of YsxC, a conserved positively charged patch including R31, R116, H117 and K146 lying adjacent to the nucleotide-binding site, and the *C*-terminal alpha helix, was investigated. *C*-terminal truncation variants of YsxC were unable to bind to both ribosome and rRNAs, whereas mutations in the other cluster did not affect YsxC binding. Our results indicate that the basic *C*-terminal region of YsxC is required for its binding to the 50S ribosomal subunit.

1. Introduction

YsxC is a GTPase that is relatively abundant in vegetative *Bacillus subtilis* cells, where 1000 YsxC molecules per cell were estimated [1]. Expression of *ysxC* is essential for cell viability in bacteria [2–5] but dispensable in *Saccharomyces cerevisiae* [3]. Essentiality of YsxC in bacteria makes YsxC a target for the design of novel antimicrobial agents. Overexpressed YsxC in *Escherichia coli* co-purifies with ribosomal material [6]. Purified YsxC preferentially binds the 50S subunit of *B. subtilis* ribosomes [6,7] and this binding occurs in the absence of nucleotides [7]. YsxC interacts in vitro with L1, L6 and L7/12 [6], as well as with rRNA [7]. Depletion of YsxC in *B. subtilis* leads to accumulation of immature ribosomal subunit [8], suggesting that YsxC is required for 50S ribosomal subunit biogenesis.

The proteins from *B. subtilis* [9,10] and *Thermotoga maritima* [11] have been crystallized. A striking feature in the YsxC structure is the presence of a cluster of positively charged residues (Arg31, Arg116, His117 and Lys146) lying at the surface of the protein and being conserved in the YsxC family [10]. Similar clusters were reported to often participate in interactions with hydroxyl groups or with ion species such as phosphate or sulfate [10].

The aim of this study was to identify the residues involved in ribosome-YsxC binding, which would help in elaborating specific inhibitors of the protein. Two basic regions were examined: one cluster located in the *C*-terminal alpha helix, and the patch of surface exposed amino-acids in the YsxC family revealed by the crystal structure of *B. subtilis* YsxC [10] (Figs. S1 and S2).

2. Materials and methods

2.1. Cloning, expression and purification of YsxC and its mutants

Recombinant *B. subtilis* YsxC and YsxC-S37A were cloned, expressed and purified as described [6,7].

Standard genetic and molecular biology techniques were used for construction of strains and plasmids [12]. Mutations in the

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cluster of basic residues (R31A, R116A, H117A, H117N, K146A, K146M) were generated by the Quickchange protocol (Stratagene) with the pET15b-*ysxC* [6] used as a template, and the oligonucleotides indicated (Table S1). *C*-terminus deleted $(His)_6$YsxC mutants, del(K179-R195), del(K182-R195), and del(K190-R195) were amplified by PCR, using YsxC NdeF primer and YsxC Bam178R, YsxC Bam181R, and YsxC Bam189R primers, respectively (see Table S1). They were ligated into the NdeI and BamHI sites of the pET-15b vector (Novagen). All constructs were sequenced (Genome express), and mutants were expressed at 18 °C and purified as for the wild-type protein [6] (see Fig. S3).

2.2. Thermal shift assay (TSA) and nucleotide binding

TSA was realized as described [7]. Fluorescence of SYPRO Orange dye was monitored as it interacts with the protein undergoing thermal unfolding. The temperature at which 50% of the protein population is unfolded or melting temperature (Tm) was taken as a reference of the thermal stability of the protein. The Thermal Shift assay was used to study binding interactions between YsxC and nucleotides. The ligand generally stabilizes the protein and the Tm of the complex is higher than that of the apo protein [13].

2.3. Enzymatic digestion of YsxC and its mutants

Limited proteolysis of the recombinant proteins (2 μg) was performed in a 100 mM $NaPO_4$-0.15 M NaCl buffer at 37 °C for various times (0–3 h) by addition of 0.1% proteolytic enzymes (subtilisin or trypsin). The digested products were separated by SDS–PAGE electrophoresis.

2.4. Molecular dynamics simulations

Molecular dynamics simulations of the apo form of YsxC (PDB: 1SUL, chain B, [10] and of the *C*-terminal mutants del(K190-R195) and del(K182-R195) were performed using the coarse-grain protein model PaLaCe [14] implemented in the Molecular Modeling Toolkit (MMTK) [15]. By adopting this model residue-specific interactions involved only one to two pseudoatoms per side-chain. At the same time an atomistic description of the backbone allowed secondary structure hydrogen bonding to be taken into account and the dynamics of folded proteins to be simulated without conformational restraints. The *C*-terminal residues from the crystallographic structure of the full-length chain were manually removed to obtain the starting structure of the two mutants. Each protein was initially relaxed (5000 steps of steepest descent followed by 500 steps of conjugate gradient energy minimization), then heated up to 300 K in 500 ps. For each system the productive phase consisted of 400 ns of Langevin dynamics, performed in the NVT ensemble at 300 K, with a time step of 5 fs and a collision frequency of 1 ps^{-1}. Conformations from the trajectories were collected for subsequent analysis every 100 ps.

2.5. Analysis of the molecular dynamics trajectories

Root mean square deviation (RMSD) and TM-score [16] were used. The convergence of each system on the ns timescale was assessed using the backbone RMSD with respect to the initial structure after heating, having checked that the heating did not induce major conformational changes (backbone RMSDs from the starting structures below 3.5 Å). The native-like shape of the wild type and mutants folds was evaluated using a TM-score. The TM-score was preferred because it is more sensitive to the fold topology than the RMSD. TM-scores range from 0 to 1, and a score above 0.5 generally means that the conformation under analysis belongs to the same fold as the reference structure [17]. Clustering of the sampled conformations was performed with the average linkage hierarchical clustering algorithm ($\varepsilon = 3$) using AMBER11 [18]. Representative structures were visualized in PyMOL (PyMOL Molecular Graphics System, Version 1.1 Schrödinger, LLC). Global compactness was measured in terms of radius of gyration, normalized by a theoretical radius of gyration for globular proteins [19].

The GDP-binding residues were defined as those having at least one non-hydrogen atom at less than 5 Å from GDP in the crystallographic structure of YsxC bound to GDP (PDB: 1SVI, [10]. The degree of burial of the binding residues was calculated in the apo forms in terms of circular variance (CV) using the coarse-grained protein model [20]. CV ranges from 0 (completely buried) to 1 (completely exposed) and, as shown earlier, is closely linked to the degree of hydration [21]. The burial profile of the GDP-binding site of the apo forms was defined as the CV value averaged over all the pseudoatoms of the binding residues, as a function of the time.

2.6. Binding of YsxC to ribosome

Ribosome purification [22] and preparation of 30S and 50S subunits have been described [6,7]. A filtration-based binding assay was used [7,23]. 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_4-Cl, 60 mM KCl, 2.5 mM 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 from Pierce. Control experiments without ribosomes were done.

2.7. rRNA blot overlay assay

In vitro synthesis of 5S, 16S, and 23S RNA and 5′-end rRNA biotinylation have been described previously [7]. rRNA blot overlay assays were performed to identify interactions between YsxC and rRNA, following the protocol described by Palaniyandi et al. [24]. In brief, YsxC proteins (wild-type or mutants) were allowed to migrate on a 14% SDS–PAGE. Proteins from the gel were electrotransferred to nitrocellulose membranes, 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).

3. Results and discussion

3.1. Obtention of YsxC mutants

The YsxC protein lacks a distinct KH domain, which is a classical RNA-binding domain [25] present in other GTPases that interact with the ribosome such as Era [26,27], or EngA [28]. The importance of the KH domain has been demonstrated for Era, since truncated Era genes were unable to complement an *E. coli* mutant strain defective in Era expression [29]. Likewise, the KH domain of EngA has been shown to be involved in the binding of EngA to the 30S ribosomal subunit [30]. Instead, YsxC possesses a highly basic *C*-terminal alpha helix that could be involved in the association with the ribosome. Truncated YsxC proteins were therefore