The Heat Shock Protein YbeY Is Required for Optimal Activity of the 30S Ribosomal Subunit

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The highly conserved bacterial ybeY gene is a heat shock gene whose function is not fully understood. Previously, we showed that the YbeY protein is involved in protein synthesis, as Escherichia coli mutants with ybeY deleted exhibit severe translational defects in vivo. Here we show that the in vitro activity of the translation machinery of ybeY deletion mutants is significantly lower than that of the wild type. We also show that the lower efficiency of the translation machinery is due to impaired 30S small ribosomal subunits.

Many heat shock proteins are chaperones and proteases that constitute the protein quality control system (4, 5, 13, 18). Recent studies demonstrated that beyond protein quality control, the heat shock response includes proteins implemented in the translation machinery (16, 17), such as FtsJ (2, 3) and Hsp15 (11).

FtsJ catalyzes methylation of U2552 in 23S rRNA (3). This modification occurs during the final steps of 50S biogenesis and is important for the structural stability of the 50S subunit (2). ftsJ deletion mutants accumulate ribosomal subunits at the expense of polysomes (2). Consequently, crude ribosome extracts prepared from ftsJ deletion mutants are far less active than wild-type preparations (3). Hsp15 recognizes and binds with high affinity to the aberrant state of the 50S subunit in complex with peptidyl tRNA positioned at the A site (10), which is more frequent at high temperatures (10). It has been proposed that Hsp15 participates in releasing the bound peptide and thereby helps recycle the 50S subunit (8, 10). Thus, heat shock proteins play a significant role both in the biogenesis of ribosomes and in the translation process.

YbeY is a 17-kDa heat shock protein, highly conserved among bacteria, that belongs to the UPF0054 family of metalloproteins (14, 21). In Aquifex aeolicus, analysis of YbeY structure homology showed similarity to eukaryotic extracellular proteases such as collagenase and gelatinase. However, in vitro experiments could not detect collagenase, gelatinase, or other hydrolase activity in YbeY (14).

Recently, we showed that ybeY deletion mutants exhibit severe translational defects manifesting by a very low level of polysomes and accumulation of free ribosomes and ribosomal subunits, indicating that most ribosomes in the cell are not engaged in translation. This translational defect intensifies at elevated temperatures (42°C) and results in growth arrest (17).

Here we present in vitro studies indicating that the activity of the translation machinery prepared from ybeY deletion mutants is lower than in the wild type. In addition, we show that this lower activity stems specifically from a defective 30S ribosomal subunit.

MATERIALS AND METHODS

Purification of ribosomes and S100 subunits. Ribosomes and S100 subunits were prepared essentially as described previously (1). Cultures of Escherichia coli MG1655 and its ΔybeY derivative were grown in LB broth (Difco) medium at 37°C to an optical density at 600 nm (OD600) of 0.5 and immediately poured onto 37°C to an optical density at 600 nm (OD600) of 0.5 and immediately poured onto an equal volume of crushed ice. Pellets were washed once with 30 ml buffer consisting of 20 mM HEPES-KOH (pH 7.5), 30 mM NH₄Cl, 6 mM MgCl₂, and 4 mM β-mercaptoethanol and resuspended in 30 ml of the same buffer. Cells were disrupted using a French press, and the lysates were centrifuged for 1 h at 4°C to 32,000 × g using a fixed-angle 70Ti rotor to remove cell debris and membranes. The upper three-fourths of the supernatant was centrifuged for 17 h at 70,000 × g at 4°C to pellet ribosomes. The top three-fourths of the supernatant was centrifuged once more for 4 h at 150,000 × g at 4°C. The top three-fourths of this supernatant was dialyzed in a dialysis bag with a molecular weight cutoff of 3,500 against 100 volumes of the same buffer for 2 h with three buffer changes. The dialyzed S100 subunit was concentrated using a centrifugal filter unit with a molecular weight cutoff of 3,500 (Amicon Ultra) until a protein concentration of 3 to 5 µg/µl was reached.

Ribosomes were prepared from the 70,000 × g ultracentrifugation pellet by the following procedure. The pellet was washed twice with 1.5 ml buffer containing 20 mM HEPES-KOH (pH 7.5), 200 mM NH₄Cl, 1 mM MgCl₂, and 4 mM β-mercaptoethanol. Next, the pellet was scraped off with a plastic sliver and completely dissolved by gentle shaking in 1.5 ml of the same buffer for 2 h at 4°C.

In order to purify the ribosomal subunits, crude ribosome extract (the dissolved 70,000 × g pellet) with an A₂₆₀ of 100 was loaded onto a 10 to 30% sucrose gradient prepared with the same buffer. The 30S and 50S subunits were separated by ultracentrifugation in an SW28 swinging-bucket rotor for 17 h at 48,000 × g at 4°C. Fractions were harvested from top to bottom, and peaks corresponding to the 30S and 50S ribosomal subunits were separately collected to avoid any cross contamination between the two species. The 30S and 50S subunits were concentrated by ultracentrifugation in a 70Ti rotor for 20 h at 190,000 × g at 4°C, and the pellet was treated as described for the 70,000 × g pellet. Ribosomes were quantified by A₂₆₀ measurements. In a typical preparation, a concentration of 0.05 to 0.2 U of A₂₆₀/µl was achieved.

In vitro transcription-translation assays. In vitro transcription-translation reactions were in a final volume of 30 µl containing 60 mM HEPES-KOH (pH 7.5); 14 mM magnesium acetate; 200 mM potassium glutamate; 30 mM ammonium acetate; 2 mM dithiothreitol; 4% PEG 8000; 1.5 mM ATP; 1 mM CTP, GTP, and UTP; 0.7 mM cyclic AMP; 80 nM creatine phosphate; 0.125 mg/ml creatine kinase (Sigma); 0.18 mg/ml total E. coli tRNA (Sigma); and 1 mM each amino acid. One hundred nanograms of pT7VEX (Roche) with a cloned hecierase gene, 10 U of T7 RNA polymerase (New England Biolabs), 0.33 A₂₆₀ U of the 30S subunit, 0.66 A₂₆₀ U of the 50S subunit, and 16.2 µg of the S100 subunit were then added to initiate the reactions. Incubation was at 37°C, reactions were stopped by the addition of 50 µl chloramphenicol at a final reaction mixture...
The lysates were cleared by centrifugation for 10 min at 20,000 g at 4°C. Pellets were resuspended in a buffer containing 20 mM HEPES-KOH (pH 7.5), 200 mM NH₄Cl, 1 mM MgCl₂, and 4 mM β-mercaptoethanol. Gentle lysis was achieved by addition of lysozyme (0.75 mg/ml final) and incubation on ice for 3 min, followed by three freeze-thaw cycles with liquid N₂.

The lysates were cleared by centrifugation for 10 min at 20,000 × g at 4°C. Thirteen A260 units were applied to 5 to 25% sucrose gradients. The lysates were separated on sucrose gradients prepared in a buffer consisting of 20 mM HEPES-KOH, 200 mM NH₄Cl, 1 mM MgCl₂, and 4 mM β-mercaptoethanol by centrifugation for 5 h at 187,000 × g at 4°C in an LE-70 ultracentrifuge (Beckman). Gradients were fractionated into 48 fractions from top to bottom and read in UV-transparent microplates (Costar), and A260 was read in a Spectra-Max 190 (Molecular Devices). Fractions were pooled and trichloroacetic acid (TCA) precipitated in order to detect YbeY, S20, and β-galactosidase (β-gal) as described below.

**FLAG tagging for immunoblotting.** Chromosomal epitope tagging was achieved using the system described by Uzzau et al. (20).

**SDS-PAGE and immunoblotting.** Gel electrophoresis was carried out according to published protocols using sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed according to standard procedures to detect YbeY and ribosomal protein S20 tagged with the FLAG×3 epitope at the C terminus. Mouse monoclonal antibodies specific for luciferase, β-gal, and FLAG×3 (Sigma) were used as primary antibodies, and horseradish peroxidase-conjugated anti-mouse IgG (Sigma) was used as the secondary antibody. The EZ-ECL chemiluminescence detection kit (Biological Industries) was used for development, and the immunoblots were scanned using an ImageScanner (Amersham Pharmacia Biotech).

**Results**

**Reduced in vitro activity of the ΔybeY translation machinery.** In vivo studies of the ΔybeY mutant (17) suggest that YbeY directly affects ribosomes or ribosome-associated factors that function in the translation machinery. This result could reflect a direct involvement of YbeY in the translation process. However, it is possible that YbeY affects the cellular translation machinery indirectly. For instance, depletion of nucleotides (7), starvation for an amino acid (15), and even a specific defect in cotranslational protein targeting of integral inner membrane proteins (6) can all lead to an apparent general translational defect.

In order to distinguish between the two alternatives, the translation machinery was prepared from wild-type and ΔybeY mutant cultures grown at 37°C and used for coupled in vitro transcription-translation assays as described in Materials and Methods. In these assays, translation efficiency was determined by measurements of the activity of the translated reporter protein—Photinus pyralis firefly luciferase. Figure 1 presents the results of luciferase activity measurements as a function of the reaction time of the translation machineries prepared from the wild type and the ΔybeY mutant. The results indicate that the activity of the wild-type system is 8- to 9-fold higher than that of the ybeY deletion mutant. This difference remains constant throughout the reaction, from 10 min to the end of the reaction after 90 min. The reaction reaches saturation after approximately 60 min in both the wild type and the ΔybeY mutant (Fig. 1).

The lower luciferase activity may indicate a lower luciferase protein level. However, it is also possible that this lower activity is due to severe misreading by the defective machinery, resulting in multiple translation errors, which lower the activity of the enzyme. To distinguish between these two possibilities, we determined the concentration of luciferase by Western blotting (Fig. 2). The data indicate that the concentration of luciferase is significantly reduced when it is translated from the ybeY machinery. These results also indicate that YbeY directly affects one of the components of the translation machinery.

**YbeY is located in the S100 fraction.** The translation machinery includes the ribosomes and the S100 fraction, which consists of all low-molecular-weight factors, such as translation factors (initiation, elongation, release, and recycling factors) and enzymes such as tRNA synthetases. In order to determine whether YbeY is in the S100 fraction or in the ribosomal fraction, we performed an analysis of ribosome profiles and detected the presence of YbeY in the various samples by im-

![FIG. 1. Reduced in vitro activity of the ΔybeY mutant translation machinery.](image-url)
FIG. 3. YbeY does not comigrate with ribosomes. Cultures of E. coli MG1655 with chromosomally FLAG-tagged (20) ybeY and rpsT were grown in LB at 37°C to an OD_{600} of 0.4 and then cooled down on ice for 15 min to allow runoff of nascent peptides. Next, lysates were prepared and 13 OD_{260} units was separated through a 5 to 25% sucrose gradient by ultracentrifugation. Gradients were fractionated from top to bottom, and OD_{260} was determined. The presence of YbeY, S20, and β-gal in the different fractions was determined by TCA concentration, SDS-PAGE separation, and immunoblotting as described in Materials and Methods.

munoblotting. The addition of a FLAG epitope-encoding sequence to the endogenous copy of ybeY on the chromosome did not compromise the ability of this strain to grow at high temperature (data not shown). Therefore, it is reasonable to assume that FLAG-tagged YbeY behaves the same as wild-type YbeY in vivo. The results presented in Fig. 3 indicate that YbeY is found entirely in the soluble protein fractions, similar to β-galactosidase and in contrast to the ribosomal protein S20 (RpsT). Therefore, we can conclude that YbeY is not associated with the ribosomes in our preparations.

The S100 fraction prepared from the ΔybeY mutant is as active as the S100 fraction prepared from the wild type. In order to determine which constituent of the translation machinery is impaired in the ΔybeY mutant, an in vitro translation assay was performed with all four possible combinations of ribosomes and S100 fractions. The results (Fig. 4) indicated that when wild-type ribosomes were supplemented with the S100 fraction prepared from the ΔybeY mutant, the luciferase activity was similar to that achieved when wild-type ribosomes were provided with the wild-type S100 fraction. This result implies that the S100 fraction prepared from the ΔybeY mutant is almost as active as the S100 fraction prepared from the wild type. The reciprocal experiment shows that low levels of luciferase activity are obtained wherever ΔybeY mutant ribosomes are present, irrespectively of the source of the S100 fraction. This result indicates that the ribosomes prepared from the ΔybeY mutant are defective.

The lower activity of the ΔybeY mutant translation machinery stems from the 30S subunit. Further purification of the ribosomal fraction preparations of both the wild type and the ΔybeY mutant enables isolation of the 30S small ribosomal subunits and the 50S large ribosomal subunits and determination of which ribosomal subunit is defective in ybeY deletion mutants. An assay with the wild-type S100 subunit and all four possible combinations of the 30S and 50S subunits was performed, and the results are presented in Fig. 5A. When wild-type the 30S fraction was supplemented with the ΔybeY mutant 50S subunit, the luciferase activity was approximately the same as the value achieved with the wild-type 50S subunit. However, addition of the ΔybeY mutant 30S subunit to the wild-type 50S subunit yielded a luciferase activity which was much lower, indicating that the 30S ribosomal subunit of the ΔybeY mutant is defective. This defect of the 30S ribosomal subunit could be shown with the S100 subunit from the ybeY deletion mutant as well (Fig. 5B).

**DISCUSSION**

The heat shock protein YbeY is highly conserved in bacteria, yet its precise function is still not known. Previously, we showed that YbeY is involved in protein synthesis. Here we show that YbeY is directly involved in the translation process and its function is essential for optimal activity of the 30S ribosome subunit.

Although YbeY is localized in our preparation within the S100 fraction (Fig. 3), its absence from the S100 fraction prepared from the ΔybeY mutant has a minor effect on this S100 fraction activity in vitro (Fig. 4). The S100 subunit provides the in vitro translation assay with all of the factors that are critical during every translation event, such as initiation, elongation, termination, and release factor and tRNA synthetases. Depletion of any of these factors from the S100 subunit should lead to a substantial decrease in translation activity. Therefore, we can assume that YbeY is not a translation factor which is required in every translation cycle. Instead, the absence of
YbeY leads to impaireld 30S ribosomal subunits (Fig. 1). As lower 30S subunit activity often stems from inefficient biogenesis, we further assume that YbeY functions in the biogenesis process of the 30S ribosomal subunit. Support for this assumption was obtained by experiments indicating that addition of purified wild-type YbeY to an in vitro translation system did not overcome the effect of the ybeY deletion mutation. These data suggest that in ybeY mutants, the 30S ribosomal subunit is defective in a way that cannot be corrected after biogenesis.

The biogenesis of the 30S ribosomal subunit involves numerous enzymes, including RNases, RNA chaperones and helicases, modifying enzymes, and small GTPases (9, 12). Some of these proteins have not been identified yet, while the function of others is poorly understood. YbeY may well be one of those proteins which are involved in the biogenesis of the small ribosome subunit and therefore essential for optimal 30S subunit activity.

The similarity of the YbeY active site to the active site of matrix metalloproteinases raises the possibility that YbeY is a peptidase or even a protease (14). At this point, it is difficult to determine how peptidase or protease activity of YbeY affects the process of 30S subunit biogenesis.

YbeY is a heat shock protein which, together with FtsJ and Hsp15, forms a group of heat shock proteins involved in the translation process (16, 17). We propose that the cellular response to heat shock in bacteria consists of two pathways. The first and major one includes the protein quality control of mature polypeptides. The second includes proteins which function in ribosome biogenesis and in the translation process. The concerted action of both pathways ensures the availability of a sufficiently large pool of functional proteins during heat shock.

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