

RECONSTITUTION AND CRYSTALLISATION EXPERIMENTS WITH ISOLATED
SPLIT PROTEINS FROM BACILLUS STEAROTHERMOPHILUS RIBOSOMESH.S. Gewitz, C. Glotz, P. Goischke, B. Romberg, J. Müssig,
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SUMMARY

Six proteins (B-L1, B-L6, B-L10, B-L11, B-L12 and B-L16) were removed from 50S ribosomal subunits of Bacillus stearothermophilus by treatment with ethanol and ammonium chloride. The proteins were isolated in a pure form, and one of them (B-L6) was crystallized. Five of the six proteins (in various combinations) were added back to the core particles, resulting in 50S subunits lacking one protein. The biological activities of these ribosomal particles as determined in the poly(U)-system varied over a wide range, depending on the protein which was omitted. The particles lacking one protein provide useful tools for heavy-atom derivation necessary for our crystallographic studies on the 50S subunits of Bacillus stearothermophilus.

INTRODUCTION

Three-dimensional crystals of 50S ribosomal subunits of Bacillus stearothermophilus and Halobacterium marismortui have been subjected to crystallographic studies (1,2). Those from halobacteria diffract to 6 Å (2). An important step for the further development of these investigations is the preparation of heavy-atom derivatives of the ribosomal particles. One of the approaches to obtain such derivatives is to produce 50S subunits in which one of the proteins is missing, to bind heavy-atom compounds to this protein and to incorporate the complex into the ribosomal particle.

A number of mutants of E. coli (3) and Bacillus stearothermophilus (4) whose 50S ribosomal subunits lack one protein have been isolated. A mutant of B. stearothermophilus in which the ribosomal protein B-L11 is missing from the 50S subunit (4) has

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been used for obtaining heavy-atom derivatives of the 50S ribosomal particles (5).

An alternative way to produce 50S ribosomal subunits in which one protein is absent is described in this paper. First the subunits were treated with ethanol/ NH_4Cl at 4° and 46°C (6,7), resulting in the removal of six proteins. Then the six proteins were isolated in a pure form, and five of them (in various combinations) were added back to the core particles. In this way, a series of 50S subunits was obtained in which one of each of the six proteins was missing. The biological activity of the various 50S particles lacking one protein was determined, and it was found to vary considerably depending on the protein which was omitted.

MATERIALS AND METHODS

Bacterial cells and ribosomes

B. stearothermophilus strains 799 or NCA 1503 were grown at 60°C to mid-log phase in L-medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 5 g glucose and 1.68 mg $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ per 1 liter. The pH was adjusted with NaOH to pH 7.2. The preparation and separation into subunits of ribosomes were as described in (8).

Extraction of protein B-L12

Protein B-L12 was selectively removed from ribosomes or 50S subunits by the ethanol/ NH_4Cl extraction method (6,7). 240 ml of 70S ribosomes or 50S subunits (200 A_{260}/ml) in TMA-I buffer (10 mM Tris-HCl, pH 7.8, 10 mM MgCl_2 , 30 mM NH_4Cl , 6 mM 2-mercaptoethanol) were mixed with an equal volume of imidazol buffer (20 mM imidazol, pH 7.5, 2 M NH_4Cl , 40 mM MgCl_2 , 1 mM 2-mercaptoethanol) and stirred at 0°C for 15 min. 240 ml of cold ethanol was added slowly over a period of 15 min. After 15 min incubation at 0°C another 240 ml of cold ethanol were added slowly, and the mixture was stirred for 10 min.

The precipitated core particles were pelleted by centrifugation for 10 min at 10 000 rpm and 4°C. The cores were extracted a second time under the same conditions with EtOH/ NH_4Cl in the cold, redissolved and dialyzed against TMA-I buffer.

2.2 volumes of acetone precooled to -25°C were added to the supernatants. After 16 hrs at -25°C the precipitated protein B-L12 was pelleted for 10 min at 10 000 rpm and 4°C, and the pellet was dissolved in 80 ml of protein buffer (10 mM sodium phosphate, pH 7.0, 30 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol) and dialyzed against the same buffer. The yield was 90 mg protein determined by the Bio-Rad protein assay (9) with bovine serum albumin as standard.

Extraction of proteins B-L1, B-L6, B-L10, B-L11, B-L16

To 39.500 A₂₆₀ of protein B-L12 depleted ribosomes (cores) in 200 ml of TMA-I buffer, 67 ml of 4 M NH₄Cl were added, and the solution was stirred for 45 min at 46°C. One volume of prewarmed (46°C) ethanol was then added slowly, and the mixture was again stirred for 45 min at 46°C. The precipitated core particles were pelleted by centrifugation for 10 min at 10.000 rpm. They were redissolved with TMA-I buffer and re-extracted at 46°C a second time in the same way as just described. The combined supernatants were mixed with 2.2 volumes of cold acetone (-25°C) and kept overnight at -25°C to complete the protein precipitation. After pelleting by centrifugation, the proteins were dissolved in protein buffer and dialyzed against the same buffer.

Isolation of the split proteins

The protein B-L12 was purified to homogeneity by chromatography on a DEAE-Sephadex A25 column (5 x 1 cm) equilibrated with protein buffer containing 30 mM NaCl. The protein was eluted with a linear gradient from 30 to 400 mM NaCl in protein buffer. Fractions of 2.5 ml were collected at a flow rate of 12.5 ml/h.

The split proteins from the 46°-extraction were separated on a CM-Sephadex C1 6B column (8 x 1 cm) equilibrated with protein buffer. A linear gradient from 30 to 600 mM NaCl was applied for the elution of the proteins. Fractions of 4.5 ml were collected at a rate of 12.5 ml/h. The proteins in the fractions were detected by the Bio-Rad protein assay (9) and by SDS-polyacrylamide gel electrophoresis (10).

Fractions containing the same protein were pooled and desalted by dialysis against protein buffer. The protein was concentrated by embedding the dialysis tube at 4°C into solid polyethylene glycol 20.000.

If necessary, gel filtration on Sephadex G50 or G75 was used for a further purification step. The proteins were identified by two-dimensional polyacrylamide gel electrophoresis in the presence or absence of about 10 µg of total 50S proteins as background.

Protein B-L10 was also isolated by HPLC chromatography. The mixture of the proteins extracted from the ribosomal particles at 46°C was applied on a Vydak C4 column (25 x 0.4 cm) and eluted with an isopropanol gradient (11).

Electrophoresis

The proteins in the fractions after column chromatography were identified by SDS polyacrylamide slab gel electrophoresis with a 15% separation gel (10).

Two-dimensional acrylamide gel electrophoresis was performed as described (12). Before electrophoresis the proteins were extracted from the ribosomal particles by 67% acetic acid (13), dialyzed against 2% acetic acid, lyophilized and dissolved in the sample loading buffer (12).

Partial reconstitution and biological activity

After their isolation the purified split proteins and the core particles were dialyzed against reconstitution buffer (20 mM Tris-HCl, pH 7.5, 20 mM Mg acetate, 0.2 mM EDTA, 400 mM NH₄Cl and 4 mM β-mercaptoethanol). The proteins were added in 2.5 molar excess to the core particles and incubated for 40 min at 60°C.

After the incubation the samples were cooled and used for determination of their biological activity in the poly(U)-system. To obtain optimal activity, 30S subunits from E. coli were added to the 50S subunits or the core particles from B. stearothermophilus.

Crystallization

Protein B-L6 was crystallized in hanging drops by vapour diffusion in Linbro dishes using 1.8 M $\text{NaH}_2\text{K}_2\text{HPO}_4$, pH 7.8, and 5% dioxane as precipitant (14).

Nomenclature

The ribosomal proteins are designated according to (15). This nomenclature is based on the sequence homology between the ribosomal proteins from Bacillus stearothermophilus and E. coli.

RESULTS AND DISCUSSION

Isolation of proteins

For isolation of the split proteins, 50S ribosomal subunits from Bacillus stearothermophilus were treated with ethanol/ NH_4Cl first at 0°C and then at 46°C. In the first step mainly protein B-L12 was extracted, and it was purified by DEAE-Sephadex chromatography (not shown). In the second step five proteins were split off from the B-L12 depleted ribosomal particles. These split proteins were identified by two-dimensional gel electrophoresis as proteins B-L1, B-L6, B-L10, B-L11 and B-L16, and they were isolated by chromatography on CM-Sephadex (Fig. 1). Since protein B-L10 always gave a rather poor yield by this separation method it was also isolated by HPLC (11). It is interesting that the same proteins were extracted regardless as to whether 50S subunits or 70S ribosomes were used. Therefore, for the isolation of the split proteins on a large scale it is not necessary to separate the ribosomes into their subunits by sucrose gradient centrifugation in zonal rotors.

Partial reconstitution

As mentioned in the Introduction, the goal of the reconstitution studies was to produce 50S ribosomal subunits in which one protein at a time is missing.

After treatment of the 50S subunits with ethanol/ NH_4Cl at 0°C, which resulted in the removal of protein B-L12, the biolo-

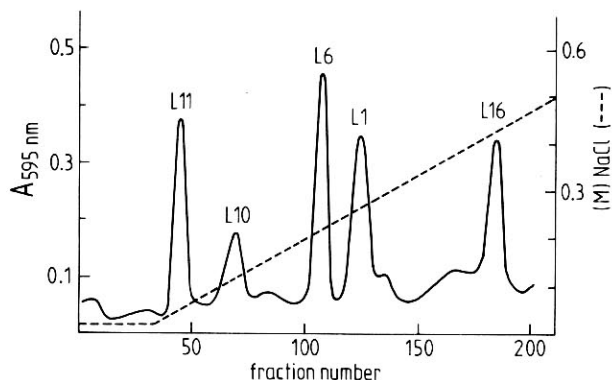


Fig. 1: Separation on a CM-Sepharose Cl 6B column of the five proteins extracted from 50S ribosomal subunits of Bacillus stearothermophilus by the second step of the ethanol/ NH_4Cl method.

gical activity of the particles in the poly(U)-system was drastically decreased to 25% (Table 1). It could however be increased back to 85% by addition of the 0°C -extract. This result confirmed the functional importance of protein L12 (6,7).

When, in addition to B-L12, the five other split proteins (B-L1, B-L6, B-L10, B-L11 and B-L16) were removed, the residual activity of the core particle was only 10% of the untreated control. However, addition of all six proteins led to the recovery of almost full (90%) activity.

Omission of one protein at a time resulted in a different decrease of the activity in the poly(U)-system, depending on the protein omitted (Table 1). When proteins B-L1 or B-L6 were omitted, this had only a small effect (75 - 80% residual activity) whereas omission of B-L12 or B-L16 led to a drastic decrease of the activity (25 - 30% residual activity). The values for protein B-L10 (45%) and B-L11 (70%) were in between those of the other two groups.

Crystallization

Isolated protein L6. After isolation of the proteins removed from the 50S subunits by ethanol/ NH_4Cl at 0°C and 46°C they were

Table 1: Biological activity of B. stearothermophilus 50S ribosomal subunits and their core particles

Particles	Protein extract added	B-L1	B-L6	B-L10	B-L11	B-L12 _A	B-L16	% Activity in poly(U)-system
50S subunits	-	-	-	-	-	-	-	100*
0°C core	-	-	-	-	-	-	-	25
0°C core	0° extract	-	-	-	-	-	-	85
46°C core	-	-	-	-	-	-	-	10
46°C core	0° and 46° extracts	-	-	-	-	-	-	100
46°C core	-	+	+	+	+	+	+	90
46°C core	-	-	+	+	+	+	+	80
46°C core	-	+	-	+	+	+	+	75
46°C core	-	+	+	-	+	+	+	45
46°C core	-	+	+	+	-	+	+	70
46°C core	-	+	+	+	+	-	+	30
46°C core	-	+	+	+	+	+	-	25

*100% activity corresponds to 21.000 cpm. The percentages are averaged and rounded figures from at least two experiments.

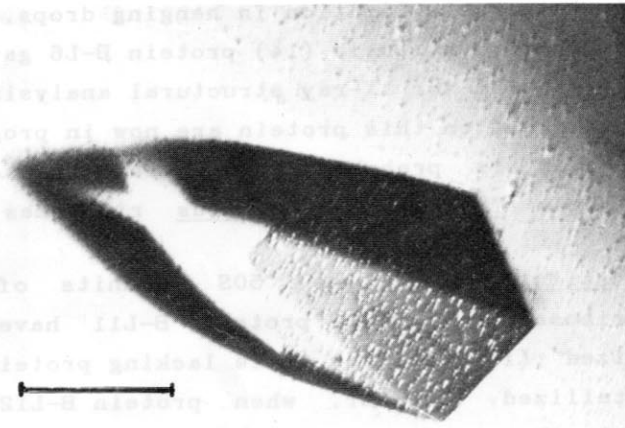


Fig. 2: Crystals of Bacillus stearothermophilus ribosomal protein B-L6. Bar: 0.1 mm.

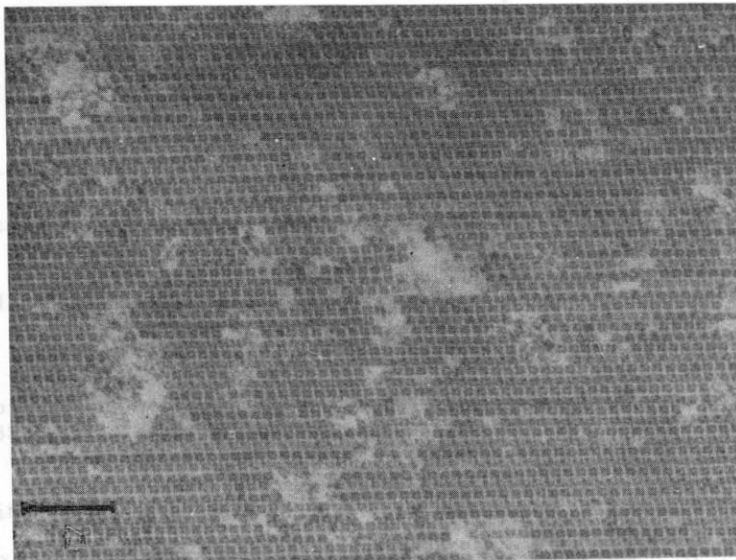


Fig. 3: Electron micrograph of a section through a crystal of Bacillus stearothermophilus 50S ribosomal subunits reconstituted by incorporation of protein B-L12 into the 50S subunit lacking B-L12. Growth conditions are described in (1). Bar: 2000 Å.

subjected to crystallization in hanging drops. Using the procedure described previously (14) protein B-L6 gave crystals (Fig. 2) large enough for X-ray structural analysis, and crystallographic studies on this protein are now in progress. Recently, a protein which is probably homologous to protein B-L6 has been isolated from Thermus thermophilus ribosomes and crystallized (16).

Reconstituted particles. 50S subunits of B. stearothermophilus ribosomes lacking protein B-L11 have previously been crystallized (17), whereas those lacking protein B-L12 could not be crystallized. However, when protein B-L12 was incorporated into the ribosomal particles lacking this protein, the reconstituted 50S subunits gave three-dimensional crystals (Fig. 3) and two-dimensional crystalline sheets. Both were isomorphous to those of native 50S subunits (1,18).

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