Characterization of Ribosomes from Dormant Spores of Bacillus cereus

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Characterization of ribosomes from dormant spores and vegetative cells of Bacillus cereus strain T has been carried out. Polyuridylic acid binding activity, ribonuclease activity associated with ribosomes, thermal denaturation profile, and sedimentation coefficients are essentially identical for both ribosomal preparations. However, ribosomal protein content of dormant spore ribosomes is about 70% of that of vegetative ribosomes. Polyacrylamide gel electrophoresis of ribosomal proteins shows that some ribosomal proteins are missing from dormant spore ribosomes. Sucrose density gradient centrifugation of ribosomes shows the existence of defective ribosomal subunits, in addition to 30S and 50S subunits, in dormant spore ribosomes. These results indicate that the ribosomes from dormant spores are distinctively different from those of vegetative cells.

The bacterial spores are one of the most typical dormant systems known and exhibit little or no metabolic activities. Protein synthesis, one of the most fundamental functions of the cells, is also non-functional in dormant spores. To understand the mechanism of dormancy in the protein synthesizing system of dormant spores of Bacillus cereus strain T, we have established a cell-free amino acid incorporating system and found that the protein synthesizing system of dormant spores possesses two defects: the one is in the supernatant, i.e., the absence, or near absence, of transfer enzyme activity, and the other is in the ribosomes, i.e., the impaired binding of aminoacyl-tRNA by spore ribosomes. On the other hand, Bishop et al.2) found that the crude extract from dormant spores of B. subtilis is capable of promoting amino acid incorporation in the presence of polyuridylic acid. The initial rate of phenylalanine incorporation was similar in extracts from vegetative cells and spores, although total incorporation was usually greater in vegetative cell extracts. Furthermore, Deutscher et al.3) found that the ribosomes from dormant spores of B. megaterium are active and apparent lack of amino acid incorporating activity may be due to the presence of high ribonuclease activity. However, Idriss and Halvorson5) compared the activity of ribosomes from spores of B. megaterium and B. cereus strain T under identical conditions and found that the ribosomes of B. megaterium had been activated during preparation of ribosomes. In the present paper, to clarify the cause of the defects in impaired aminoacyl-tRNA binding activity of dormant spore ribosomes of B. cereus strain T, precise comparisons of ribosomes from dormant spores and vegetative cells have been described.

MATERIALS AND METHODS

Microorganism. Bacillus cereus strain T was used throughout the experiments. Vegetative cells and spores were cultivated as described previously.1) Spores were germinated in the presence of L-alanine and adenosine.5)

Preparation of cell-free extracts and polyuridylic acid (poly U)-dependent 14C-phenylalanine incorporation experiments. Cell-free extracts of vegetative cells were prepared by glass beads grinding, and those from dormant and germinated spores were prepared either by glass beads grinding (Fig. 1, Table I) or by Braun cell homogenizer as described previously.5) The
conditions used for amino acid incorporation were the same as described previously.\textsuperscript{1,11}

\textbf{\textit{\textsuperscript{14}C-Poly U binding with ribosomes.}} Ribosomes from dormant spores or vegetative cells suspended in the standard buffer (0.01 M Tris-HCl, pH 7.6, 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M mercaptoethanol) were mixed with \textsuperscript{14}C-poly U (0.01 \textmu Ci), incubated in an ice-bath for 10 min, and layered on top of 5 to 20\% sucrose density gradient containing standard buffer (total volume, 5 ml). After centrifugation for 80 to 90 min at 35,000 to 40,000 rpm (as indicated in Fig. 1) in Hitachi RPS 40 rotor, fractions were collected directly in vials. A 0.02 ml-portion of a fraction was taken from the vial and O.D.\textsuperscript{260} was measured after dilution with distilled water. Ten ml of Triton X-100 (Packard Instrument Co., Inc.) were added to each vial and the radioactivity was measured in a Packard liquid-scintillation spectrometer.

**Ribonuclease activity.** Assays were carried out as follows: 0.01 \textmu Ci (0.01 ml) of \textsuperscript{14}C-poly U, 248 \textmu Ci/\textmu mole, was added to 0.24 ml of the reaction mixture containing the following (\textmu moles): Tris-HCl buffer, pH 7.6, 10; magnesium acetate, 2.4; NH\textsubscript{4}Cl, 10; GTP, sodium salt, 0.05; ATP, sodium salt, 0.2; phosphoenolpyruvic acid, sodium salt, 1.0; mercaptoethanol, 10; spermidine-3HCl, 0.2; 20 L-amino acids (minus only L-phenylalanine), 0.01 each; L-phenylalanine-U-\textsuperscript{3}H\textsubscript{14}C (100 \textmu Ci/\textmu mole), 0.0005; 3.4 \textmu g of pyruvate kinase; 3.5 O.D.\textsuperscript{260} units of B. cereus tRNA; 25 \textmu g of cold poly U; and the indicated amount of supernatant protein (S-105) or ribosomes. The reaction mixture was incubated at 36\textdegree C for 30 min, then 0.1 ml of 0.2\% yeast RNA and 1 ml of cold absolute ethanol were added. The mixture was kept in an ice-bath for 15 min and then centrifuged. A 1 ml aliquot of the supernatant solution was added into a vial containing 10 ml of Triton X-100 and the radioactivity was measured in a Packard liquid-scintillation spectrometer.

**Preparation of NH\textsubscript{4}Cl- or trypsin-treated ribosomes.** (A) NH\textsubscript{4}Cl-washed ribosomes. After disruption cells were extracted with standard buffer containing 0.5 M NH\textsubscript{4}Cl. Ribosome pellets were obtained from crude cell-free extracts (S-30) by high speed centrifugation (105,000 \times g, 90 min). Ribosomes were washed once with standard buffer and resuspended in the buffer. Undissolved material was sedimented by centrifugation at 10,000 \times g for 10 min and the supernatant was used as ribosome suspension. (B) Trypsin-treated ribosomes. Ribosome suspension in standard buffer containing 100 \textmu g of crystalline trypsin/ml was incubated for 30 min at 30\textdegree C. The incubation mixture was then layered on top of 0.15 M sucrose in standard buffer and centrifuged at 105,000 \times g for 90 min. The pellets were washed once with standard buffer and ribosome suspension was prepared as described above.

**Measurement of thermal denaturation profile of ribosomes.** Thermal denaturation of ribosome suspension (about 0.5 O.D.\textsuperscript{260} unit/ml) in fresh standard buffer was followed in a Gilford, model 2000, spectrophotometer equipped with a heated cell compartment, Haake TP 32-PG 11, and a recorder. Temperature was raised 1\textdegree C for each 3 min.

**Analytical ultracentrifugation.** Ribosome suspension of various concentration in fresh standard buffer was spun at 43,700 rpm at about 20\textdegree C in a Hitachi model UCA analytical ultracentrifuge equipped with Schlieren optics. Pictures were taken every 4 min after reaching the speed. All runs were made in a 12-mm standard cell. The sedimentation coefficients were corrected to 20\textdegree C, water and zero concentration.

**Preparation of ribosomal proteins and polyacrylamide gel electrophoresis.** Ribosomal proteins were prepared by acetate method and polyacrylamide gel electrophoresis was carried out as described previously.\textsuperscript{5}

**Sucrose density gradient centrifugation of ribosomes.** Crude extracts of adenine-8\textsuperscript{14}C labeled vegetative cells and uridine-5\textsuperscript{3}H labeled dormant spore ribosome suspension were mixed and layered on top of 5 to 20\% sucrose density gradient in 0.01 M Tris-HCl, pH 7.8, containing 0.1 mm magnesium acetate and centrifuged at 22,000 rpm for 18 hr at 4\textdegree C in a Spinco SW 25.3 rotor. After centrifugation samples were collected and the radioactivity of each fraction was measured as described previously.\textsuperscript{5}

**Determination of protein.** Protein was determined by the method of Lowry et al.\textsuperscript{5} Bovine serum albumin was used as the standard.

**Chemicals.** Uridine-5\textsuperscript{3}H (5 Ci/mm) and adenine-8\textsuperscript{14}C sulfate were obtained from Radiochemical Centre, Amersham, England and L-phenylalanine-U-\textsuperscript{3}H\textsubscript{14}C was obtained from Daiichi Pure Chemicals, Co., Ltd. \textsuperscript{14}C-Poly U (248 \textmu Ci/\textmu mole, 1 \textmu Ci/ml) was obtained from Miles Laboratories Inc., U. S. A.

**RESULTS**

**Polyuridylic acid (Poly U) binding activity of dormant spore ribosomes.**

As already shown in our previous paper,\textsuperscript{1} phenylalanyl-tRNA binding ability of dormant spore ribosomes is extremely low. However, whether the defect is due to directly an impairment of the aminoacyl-tRNA or due to the lack of messenger RNA binding ability, which causes the impaired binding of aminoacyl-tRNA, or both, remained undetermined.
FIG. 1. $^{14}$C-Poly U Binding with Ribosomes.

12.5 O.D.260 units of vegetative ribosomes (VR) or 7.3 O.D.260 units of dormant spore ribosomes (DR) was incubated with 0.01 ml ($0.001$ μCi) of $^{14}$C-poly U in an ice-bath for 10 min. Total vol., 0.2 ml. Ribosomes from vegetative cells or dormant spores were centrifuged at 35,000 rpm for 90 min (VR) or at 40,000 rpm for 80 min (DR), respectively. O.D.260 (—); counts/min (——).  

**TABLE I. RIBONUCLEASE ACTIVITY OF CELL-FREE PREPARATION OF B. cereus STRAIN T**

The following amounts of O.D.260 units of ribosomes or proteins from supernatant fraction (S-105) were used: Ribosomes from dormant spores, 2.0; germinated spores, 2.0; and vegetative cells, 2.0; supernatant fraction from dormant spores, 94 μg; germinated spores, 112 μg; and vegetative cells, 110 μg.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>14C-Poly U hydrolyzed (nmoles/μg protein/hr)</th>
<th>14C-Phenylalanine incorporated (nmoles/mg ribosomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ribosomes</td>
<td>Supernatant</td>
</tr>
<tr>
<td>Dormant spores</td>
<td>0.28</td>
<td>2.4</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>0.28</td>
<td>2.8</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>7.6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

To make clear this point, $^{14}$C-poly U binding ability of dormant spore ribosomes was measured. About 18% and 15% of the added $^{14}$C-poly U bind to ribosomes from vegetative cells and dormant spores, respectively. By addition of two-fold poly U, the amount of poly U bound to ribosomes increases two-fold, suggesting that the ribosomes possess the messenger RNA binding ability. The relatively low messenger binding ability might be due to the quality of $^{14}$C-poly U and ribosomes. Under exactly same condition, **E. coli** Q13 ribosomes bind about 36% of $^{14}$C-poly U added (Kobayashi, unpublished data). As can be calculated from Fig. 1, the counts of $^{14}$C-poly U bound per one O.D.260 unit of 70S dormant spore ribosomes are almost equal to those of vegetative ribosomes. This indicates that the $^{14}$C-poly U binding ability of dormant spore ribosomes is essentially the same as that of vegetative ribosomes.

**Ribonuclease activity of dormant spore ribosomes**

Deutscher et al.3) found that the high ribonuclease activity associated with dormant spore ribosomes was the cause of the apparent defect of dormant spore ribosomes of *B. megaterium* in protein synthesis. In order to check the possibility, ribonuclease activity

**TABLE II. EFFECT OF NH$_4$Cl-WASHING OR TRYPsin-treatment ON DORMANT SPORE RIBOSOMES**

Poly U-dependent $^{14}$C-phenylalanine incorporating activity of ribosomes was measured as described previously.5)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vegetative ribosomes</th>
<th>Spore ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.4</td>
<td>0.4</td>
</tr>
<tr>
<td>NH$_4$Cl-washing</td>
<td>14.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Trypsin (100 μg/ml)</td>
<td>1.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**FIG. 2. Thermal Denaturation Profile of Ribosomes.**

Ribosomes from: (a) dormant spores, (b) germinated spores, and (c) vegetative cells.
of ribosomes and supernatant of *B. cereus* strain T was measured under experimental conditions used for *in vitro* protein synthesis. As shown in Table I, ribonuclease activity of dormant spore ribosomes is about 1/30 of that of vegetative ribosomes and that of dormant spore supernatant is about 1.6-fold of that of the vegetative supernatant. This result suggests that the ribosome-bound ribonuclease activity is not a cause of a defect in protein synthesis in dormant spore ribosomes of *B. cereus*.

**Effect of NH₄Cl-washing or trypsin-treatment on dormant spore ribosomes**

There is a possibility that the inactive dormant spore ribosomes are masked with a protein inhibitor as observed in ribosomes from unfertilized sea urchin eggs⁷) and zoospores of an aquatic fungi.⁸) In order to test the possibility, dormant spore ribosomes were either washed with 0.5 M NH₄Cl or treated with trypsin⁹) at 30°C for 30 min. As shown in Table II, the activity of dormant spore ribosomes increased slightly by NH₄Cl-washing, but the activity is still extremely lower than that of vegetative ribosomes. Treatment of trypsin decreased the activity drastically, indicating the digestion of ribosomes by the enzyme. This result suggests that neither washing with NH₄Cl nor treatment with trypsin cannot activate the dormant spore ribosomes.

**Thermal denaturation profile of ribosomes from vegetative cells and dormant spores**

To investigate a possible difference in conformation, thermal denaturation profile of ribosomes from vegetative cells and spores was compared. As shown in Fig. 2, the Tm of the vegetative ribosomes is almost the same as that of dormant spore ribosomes. This result suggests that there is no great conformational difference between ribosomes from vegetative cells and spores. An electron microscopic observation of ribosomes (Kobayashi, unpublished result) also indicates that there is not a significant conformational difference between ribosomes from vegetative cells and dormant spores. However, the possibility of the existence of a subtle conformational difference between ribosomes cannot be excluded, since the change in relative absorbancy of the vegetative ribosomes is higher than that of the dormant spore ribosomes.

**Analytical ultracentrifugation of ribosomes**

In order to determine whether there is a difference in sedimentation coefficient of ribosomes from vegetative cells and from spores, analytical ultracentrifugation of ribosomes was carried out. Sedimentation coefficients, \( s_{20,w} \), were determined using various concentration of ribosome suspension in fresh standard buffer. As shown in Fig. 3-1, vegetative cells contain four classes of ribo-

![Fig. 3. Analytical Ultracentrifugation of Ribosomes.](image)
Characterization of Spore Ribosomes

Sucrose density gradient analysis of ribosomes was carried out in the presence of 0.1 mM magnesium acetate in order to characterize the ribosomal subunits. Figure 4 shows the presence of unusual subunits (21S, 26S, and 38S) in dormant spore ribosomes in addition to normal 30S and 50S subunits, while vegetative cells contain only 30S and 50S ribosomal subunits. Although these defective subunits are always present in dormant spore ribosome preparation, the amount of these subunits varied from batch to batch. The detailed characterization of these ribosomal subunits has been published.1

**Ribosomal protein of ribosomes from vegetative cells and dormant spores**

The presence of defective subunits in spore ribosomes suggests that the dormant spore ribosomes may contain less amount of ribosomal proteins than vegetative ribosomes. Therefore, ribosomal protein content of ribosomes from spores and vegetative cells was measured. As shown in Table III, dormant spore ribosomes contain 20.7 µg of ribosomal proteins/O.D.260, while ribosomes from vegetative cells contain 31.7 µg/O.D.260. Ribosomes from germinated spores contain 26.7 µg/O.D.260. The lack of some ribosomal proteins from dormant spore ribosomes is clearly shown by polyacrylamide gel electrophoresis. As shown in Fig. 5, several ribosomal proteins are missing from dormant spore ribosomes. When the ribosomes were prepared by NH₄Cl-washing, essentially the same distribution pattern of the ribosomal proteins was obtained.

These results and the in vitro activation of

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**Table III. Ribosomal Protein Content of Ribosomes from Spores and Vegetative Cells**

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Protein, µg/O.D.260</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>20.7 ± 2.9a</td>
</tr>
<tr>
<td>Germinated spores</td>
<td>26.7 ± 0.2b</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>31.7 ± 2.1c</td>
</tr>
</tbody>
</table>

a Average of 6 samples.  
b Average of 2 samples.  
c Average of 7 samples.
FIG. 5. Polyacrylamide Gel Electrophoresis of Ribosomal Proteins.
Ribosomal proteins from: (1) dormant spores, 190 µg and (2) vegetative cells, 180 µg.

dormant spore ribosomes by the addition of ribosomal proteins,\textsuperscript{5}) suggest that one of the major causes of the impaired aminoacyl-tRNA binding activity of dormant spore ribosomes is the lack of some ribosomal proteins. There is, however, a possibility that dormant spore ribosomes have some defects in the ribosomal RNA, causing the deletion of some ribosomal proteins. An preliminary experiment shows that ribosomal RNAs from vegetative cells and spores have essentially the same sedimentation characteristics. More detailed characterization of ribosomal RNA is now in progress.

DISCUSSION

Dormant spore ribosomes have extremely low amino acid incorporating activity. From the results presented in our previous paper,\textsuperscript{1) it is indicated that the impaired aminoacyl-tRNA binding ability is the cause of the defect in protein synthesizing activity of the dormant spore ribosomes. However, the cause of the defects in aminoacyl-tRNA binding ability has remained obscure.

The fact that the defect in dormant spore ribosomes is repaired during germination without \textit{de novo} synthesis of protein\textsuperscript{1)} suggests that the cause of the defects in aminoacyl-tRNA binding ability of dormant spore ribosomes might be due to: (1) the presence of an inhibitor or a masking substance, (2) inactive conformation of ribosomes, or (3) lack of some essential components. The presence of an inhibitor, such as ribonuclease or a masking substance, is known in several systems.\textsuperscript{2,3,7-10) However, in \textit{B. cereus} this might not be the case, since the addition of dormant spore ribosomes to the \textit{in vitro} protein synthesizing system of vegetative system did not cause the inhibition,\textsuperscript{1) and the ribonuclease activity of dormant spore ribosomes is extremely low and washing with high salt did not activate ribosomes (Tables I and II). Results shown in Figs. 2 and 3, together with the fact that the defective ribosomes can be activated by incubation with ribosomal proteins from vegetative ribosomes at 0°C,\textsuperscript{1) suggest that the second possibility also might not be the case. The presence of defective ribosomal subunits and the low protein content in dormant spore ribosomes (Figs. 4 and 5; Table III) strongly suggest that the lack of some ribosomal proteins of the dormant spore ribosomes is a major cause of the defect in aminoacyl-tRNA binding ability. These missing ribosomal protein might constitute the aminoacyl-tRNA binding site itself or have a close relationship in the construction of the binding site.

The conclusion of the present paper is different from the results obtained using other species of \textit{Bacillus}. Bishop \textit{et al.}\textsuperscript{3) found that spore extracts of \textit{B. subtilis} are capable of promoting polypeptide synthesis at almost the same rate as vegetative cell extracts on the basis of ribosome content. In \textit{B. subtilis} the cell-free amino acid incorporating activity of vegetative cells is about 1/7～1/10 of that observed for \textit{in vitro} protein synthesizing system of \textit{E. coli} and \textit{B. cereus} and amino acid incorporation levels off within 10 min.
Characterization of Spore Ribosomes

after the initiation of the reaction, suggesting a presence of an unknown inhibitor in the vegetative system. Since the rate of polypeptide synthesis in dormant spore extract is almost the same, or lower, as that of vegetative cells, it is likely that any difference between them could be masked by inactivation of both. Further characterization of the in vitro system will be necessary to reach a final conclusion in B. subtilis. Deutscher et al. also found that the ribosomes from dormant spores of B. megaterium are active and apparent lack of amino acid incorporating activity may be due to the presence of high ribonuclease activity. Idriss and Halvorson, however, showed that spores have been activated during preparation of ribosomes by the method used by Deutscher et al., whereas ribosomes prepared by mechanical disruption have very low amino acid incorporating activity. Since the active ribosomes are obtained by mechanical disruption from vegetative cells, it is unlikely that mechanical disruption inactivates dormant spore ribosomes. These results also suggest the possibility that dormant spore ribosomes of B. megaterium may be defective in protein synthesis.

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REFERENCES