ALTERATION OF THE ACIDIC RIBOSOMAL PROTEINS FROM DORMANT SPORES OF *BACILLUS SUBTILIS*

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1. Introduction

Ribosomal proteins L_7 and L_{12} of *Escherichia coli* play an important role in protein synthesis. They are required for the GTPase action of ribosomes at elongation [1,2] and initiation [3] steps and they also seem to participate in the termination of protein synthesis [4]. Similar acidic ribosomal proteins have also been identified in other organisms [5–7].

The relative levels of these acidic proteins vary during the growth cycle of *E. coli* [8,9] and they also seem to change during the development of *Artemia salina* cysts [7], suggesting that the physiological state of the cell regulates the relative amounts of these proteins in the ribosome [8,9].

During bacterial sporulation the cells undergo drastic physiological state changes. We therefore decided to examine the acidic ribosomal proteins of ribosomes from vegetative cells and dormant spores of *Bacillus subtilis*. It was found that vegetative ribosomes contain acidic proteins that migrate like *E. coli* L_7-L_{12} in polyacrylamide gel electrophoresis. When these proteins are removed from the ribosomes there is a decrease of their ability to synthesize polyphenylalanine in an in vitro system. However, the activity is completely restored when the acidic proteins are added back.

Spore ribosomes on the contrary, do not show proteins equivalent to L_7-L_{12} in polyacrylamide gels and their reduced activity for polyphenylalanine synthesis is not stimulated by the presence of vegetative state ribosomal acidic proteins.

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2. Materials and methods

Bacillus subtilis 168 (Marburg) was grown with aeration at 37°C in medium G [10]. Vegetative cells were harvested at mid-log phase (A_{660} = 1.5) and spores after 36 h of incubation in the same medium. Both, spores and vegetative cells were washed with 1 M KCl buffer to reduce proteases [11]. The spores were further treated with lysozyme and SDS [12], washed with distilled water until no SDS was present, and lyophilized. Dormant spores were disrupted in a Bronwill homogenizer (MSK). Spores (5 g) were suspended in 30 ml of buffer A (50 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA, 6 mM 2-mercaptoethanol, 3.5 mM phenylmethyl sulfonyl fluoride (PMSF) and 5% glycerol) in the presence of 60 g of glass beads (100 μm diam.) and 8 mg of makaloid per gram of cells. The homogenizer was run for 1-minute periods for a total of 10 min with intermittent cooling. The spore extract was then obtained by centrifugation of the homogenate for 20 min at 20 000 × *g*. Vegetative cells were disrupted in the Bronwill homogenizer as described for the spores or by grinding them with alumina followed by resuspension in buffer A and centrifugation for 20 min at 20 000 × *g*.

Spore and vegetative cell extracts were treated with 8 μg/ml of RNAase-free DNAase, incubated for 10 min at 37°C and then centrifuged for 30 min at 30 000 × *g* (S-30). The ribosomes were obtained by centrifugation of the S-30 for 2 h at 200 000 × *g* (high-speed centrifugation). The ribosomal pellets were then resuspended in high-salt buffer (10 mM Tris–HCl, pH 7.5, 10 mM magnesium acetate,

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0.5 M NH_4Cl and 6 mM 2-mercaptoethanol) and were centrifuged for 15 min at 20 000 X *g* (low-speed centrifugation). The high- and low-speed centrifugations in high-salt buffer were repeated twice and, finally, the ribosomal pellets were resuspended in the same buffer except the concentration of NH_4Cl was 60 mM. Before use the first high-speed supernatants were dialyzed against buffer A without PMSF.

The acidic ribosomal protein fraction of *B. subtilis* (PE) was extracted by the ethanol method [1] and was used without further purification. Ribosomes lacking PE will be called extracted ribosomes. Total ribosomal proteins were extracted by the acetic acid method [13].

Two-dimensional gel electrophoresis was carried out essentially as described by Kaltschmidt and Wittmann [14]. Analysis of the acidic proteins was done by gel electrophoresis at pH 5.0 [8] using citrate–phosphate buffer. Stained gel rods were scanned at 530 nm in a Canalco Model G densitometer. For determination of radioactivity the gels were sliced and counted in a toluene-triton X-100-based scintillation fluid.

E. coli tRNA was aminoacylated with [^{14}C]phenylalanine [15]. The extent of polyphenylalanine synthesis was determined by standard procedures [16] and the reaction mixtures were as described in the figure legends.

3. Results

When total ribosomal proteins from vegetative cell ribosomes are analyzed by electrophoresis in 4% acrylamide, pH 5.0 gels (fig.1a) it can be seen that a fast migrating band moves ahead of the rest of the ribosomal proteins. This band apparently contains both acidic proteins since it coincides with PE proteins extracted from *B. subtilis* ribosomes (solid line, fig.1c) and with a 1:1 mixture of tritiated L_7-L_{12} of *E. coli* (filled circles, fig.1c) indicating that the acidic proteins of *B. subtilis* behave like those of *E. coli* under these conditions. However, total ribosomal proteins from dormant spore ribosomes show a different pattern. The band characteristic for the acidic proteins is absent (fig.1b).

Dormant spore ribosomal proteins were also compared with those from the vegetative state using two-dimensional polyacrylamide gels. The results are shown in fig.2. In this system, the acidic proteins of *B. subtilis* migrate where indicated in the upper left corners of the plates and usually give a very faint spot in *Bacillus* ribosomes [5,17]. However, a good part of these proteins is retained on the 1-D gel rod and, as fig.2 shows (arrows), in the spore ribosomes there are no acidic proteins present. No new spots resulting from modification of the acidic proteins could be detected

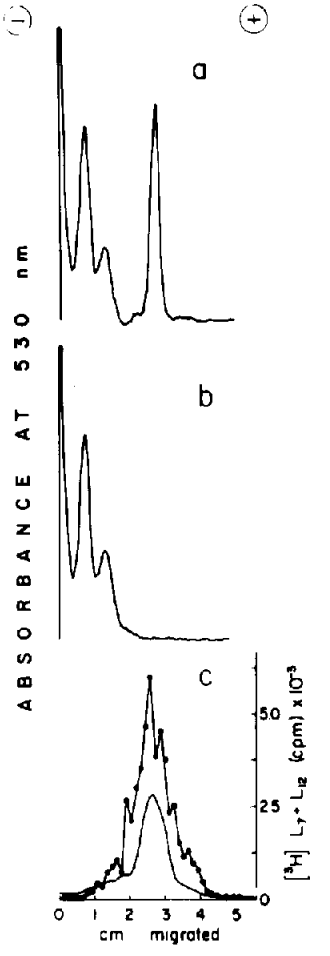

Fig.1. Analysis of acidic ribosomal proteins from different ribosome preparations. Polyacrylamide gel electrophoresis at pH 5.0 was carried out as described in Materials and methods. (a) 200 μg of total ribosomal proteins from the vegetative state was applied to the gel. (b) 200 μg of total ribosomal proteins from dormant spore ribosomes. (c) 25 μg of PE from the vegetative state were applied together with tritiated L_7-L_{12} (a total of 51 000 cpm). (———) A_{530} nm, (•———•) cpm of tritiated L_7-L_{12}.

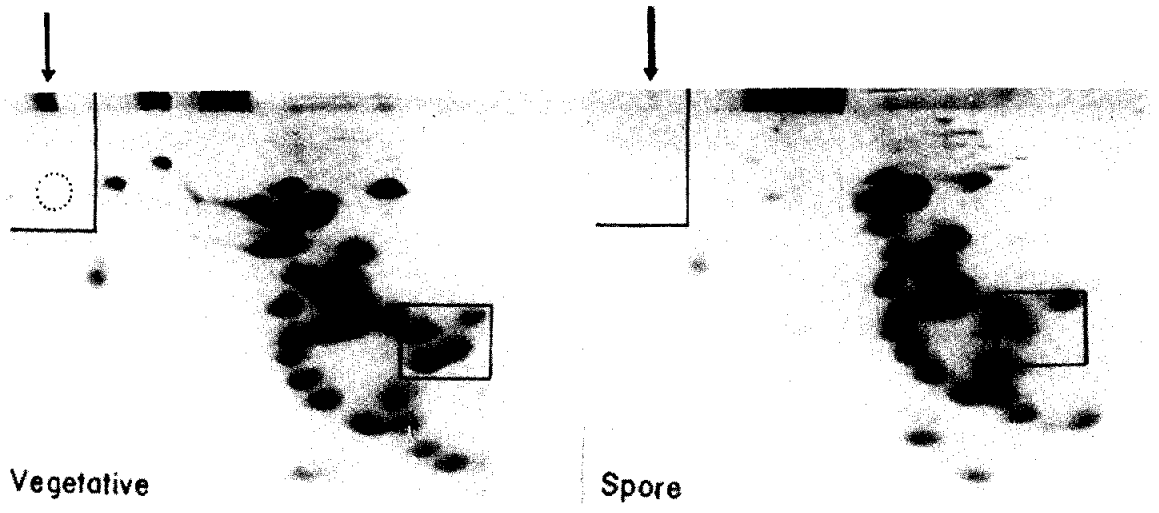

Fig.2. Two-dimensional gel electrophoresis of vegetative and dormant spore total ribosomal proteins. Electrophoresis was carried out as described in Materials and methods. The enclosed areas are described in the text.

under these conditions. Analysis of the ribosomal proteins of separated subunits will be necessary to clarify this. Figure 2 also shows that the general pattern of the ribosomal proteins does not change in the dormant spore, except for the spots enclosed by squares. These changes however, occur during the early stages of the sporulation process and we find they do not seem to affect the activity of the ribosomes in vitro [18].

It is known that dormant spore ribosomes have very low activity for protein synthesis [19–21] and this is probably a consequence of defects not only in the ribosomes themselves but also in the protein factors [21]. To find out if the reduced activity of spore ribosomes is due to the apparent change in the acidic proteins, a reconstitution experiment was done. Figure 3 shows that extracted *B. subtilis* vegetative ribosomes, i.e., lacking PE, have a decreased activity for phenylalanine polymerization. However, when PE proteins are added, a total recovery of the activity is observed as has been described for *E. coli* ribosomes [1].

The control vegetative ribosomes are not affected in their activity in the presence of the same amounts of PE. The dormant spore ribosomes however, are not stimulated by the presence of PE from the vegetative state as shown in fig.3. The results were the same when the spore ribosomes were previously extracted

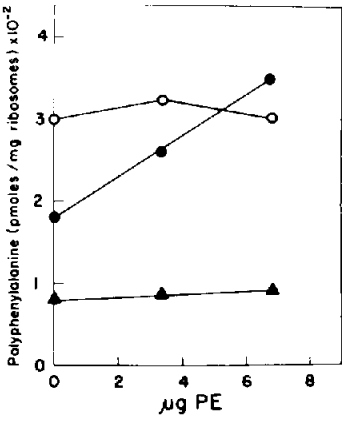

Fig.3. Effect of increasing amounts of PE proteins on the activity of ribosomes. Polyphenylalanine synthesis was assayed in 125 µl reaction mixtures containing 72 mM Tris–HCl, pH 7.5, 72 mM NH_4Cl, 12 mM magnesium acetate, 2.4 mM DTT, 2 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, 0.6 mM EDTA and 5 mM spermidine. Each assay also contained 5 µg creatine phosphokinase, 100 µg poly(U), 50 pmol [^{14}C]phenylalanyl-tRNA (1 pmol: 132 cpm), 235 µg of vegetative high-speed supernatant protein, the amounts of PE indicated and 70 µg of ribosomes. After incubation for 30 min at 37°C the extent of polyphenylalanine synthesis was determined as described in Materials and methods. (○———○) Vegetative state ribosomes; (●———●) extracted vegetative state ribosomes; (▲———▲) spore ribosomes.

with ethanol (not shown). In all of these assays, vegetative high-speed supernatant was used so that only the possible defects of the spore ribosomes were detected.

4. Discussion

Our results clearly show an alteration of the acidic protein fraction in the dormant spore ribosomes. These findings could be due to lack of acidic proteins in the ribosomes or due to modifications of these proteins altering their mobility in gel electrophoresis.

A lack of acidic proteins could be a consequence of the conditions used in isolating the ribosomes. However, we feel this is not the case since spore ribosomes were prepared under the same conditions used for vegetative ribosomes which are active in protein synthesis. The lack of PE proteins is unlikely to be due to the presence of proteases in the spores since these were extensively washed in 1 M KCl to remove exoproteases and the cell breakage was done in the presence of PMSF, a protease inhibitior [11].

It has been described that *B. licheniformis* ribosomes lose acidic proteins upon dissociation at low magnesium concentration [22]. The same could happen in the spore ribosomes by chelation of the magnesium ion due to the high dipicolinic acid content of the spore. If this loss of acidic proteins was the cause of the low activity of the spore ribosomes one would expect restoration or stimulation of the activity of these ribosomes in the presence of PE from vegetative state organisms but this was not so. Preliminary experiments using PE mixed with tritiated L_7-L_{12} of *E. coli* to measure the binding of these proteins to the ribosomes indicate that spore ribosomes do not bind them whether the ribosomes were extracted with ethanol or not. This would indicate that there is a modification of the spore ribosomes preventing the binding of acidic proteins or more likely that the acidic proteins or more likely that the acidic proteins are present in the ribosomes in a modified form, being unextractable by ethanol and preventing the binding and stimulation by vegetative state PE.

Our results might explain why dormant *Bacillus* spores show very low levels of protein synthesis [19–21]. The modification of the acidic ribosomal proteins could also constitute a late kind of control of the sporulation process since these changes start to occur at the end of the sporulation sequence [18]. In this connection, several recent reports indicate that translational controls may be important in regulating sporulation [23–26]. Studies to establish the kind of modification of the acidic protein fraction and the significance this might have in the sporulation events are in progress.

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