Binding of Ribosomes to Cytoplasmic Reticulum of

Bacillus megaterium

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ABSTRACT

Schlessinger, David (Washington University School of Medicine, St. Louis, Mo.), Vincent T. Marchesi, and Benjamin C. K. Kwan. Binding of ribosomes to cytoplasmic reticulum of Bacillus megaterium. J. Bacteriol. 90:456-466. 1965.—As many as 60% of the cellular ribosomes are bound to membrane "ghosts" in lysozyme lysates in 0.02 m Mg++. Bound ribosomes labeled with C14-uracil do not exchange with added unlabeled ribosomes, even after disruption of the cell membrane by sonic treatment. Electron micrographs of thin sections of ghosts, or of fragments produced by sonic disruption of protoplasts, indicate that the ribosomes are distributed on a reticular matrix which extends throughout the cytoplasm. The binding of ribosomes to this matrix is insensitive to ribonuclease or deoxyribonuclease, and has many other features in common with the binding of ribonucleoprotein to the membranous elements of the mammalian microsomal fraction, though the reticulum does not appear to be membranous. Thus, functioning ribosomes may be bound to a cytoplasmic structure in all cell types.

Ribosomes in bacteria are generally thought to exist free in the cytoplasm, in contrast to their association with a membranous reticulum in mammalian cells (for review, see Murray, 1960). However, it has been observed recently that in lysates of Bacillus megaterium most of the capacity to incorporate amino acids into protein resides in polyribosomes bound to membrane "ghosts" (Schlessinger, 1963). The association of the ribosomes with ghosts suggested that they were either structurally bound to the membranes or trapped within them as a consequence of cell lysis.

To investigate the possibility that ribosomes may exist bound to some structure within intact bacterial cells, electron micrographs have been taken of ghosts and fragments of ghosts and their associated ribosomes. The ribosomes appear to be distributed on a reticular framework which extends throughout the cytoplasm of the protoplasts. The association between ribosomes and reticulum is magnesium-dependent, insensitive to deoxyribonuclease and ribonuclease, and stable enough to withstand sonic treatment. The binding is firm enough so that, in suspensions of fragments, ribosomes associated with reticulum do not exchange for added free ribosomes.

These results suggest that ribosomes may be bound to a reticular matrix or network within intact bacteria.

MATERIALS AND METHODS

Preparation of subcellular fractions. B. megaterium KM was harvested during exponential growth at 30 C, washed at 0 C, and lysed with lysozyme at 20 C in 0.01 m tris(hydroxymethyl)aminomethane (Tris), pH 7.5. Amounts of 1 μg/ml of deoxyribonuclease 0.01 m NaCl, and Mg++ were added as required (Schlessinger, 1963). All subsequent steps were at 0 to 5 C unless otherwise specified. When membranes containing C14-labeled ribosomes were to be used, growing cells were labeled for at least three generations with 10 μc of C14-uracil per liter (290 μc/mg, from New England Nuclear Corp., Boston, Mass.).

The lysate was centrifuged for 15 min at 8,000 X g to yield the crude extract and a pellet which, after resuspension and four washes, was used as ghosts. The crude extract was centrifuged in a Spinco model L ultracentrifuge at 95,000 X g for 100 min in 4-ml tubes to yield a pellet of ribosomes. Ribosomes were washed twice more by centrifugation before use.

In experiments designed to measure exchange of free for membrane-bound ribosomes, ghosts (or sonic-treated fragments of ghosts) labeled with C14-uracil were suspended in buffer or in solutions containing free, unlabeled ribosomes. At intervals, 0.05-ml samples were again separated into membranes and supernatant fluid by centrifugation for 5 min at 12,000 X g in capped hemocytor tubes, by use of a microcapillary centrifuge (model MB; International Equipment Co., Boston, Mass.). Ghosts containing essentially no ribonucleic...
acid (RNA; as measured by ultraviolet absorption or the orcinol reaction; see Schlessinger, 1963) were prepared by mixing a suspension with an equal volume of 0.1 M ethylenediaminetetraacetate (EDTA)-0.1 M Tris (pH 7), and then, after 10 min at 0°C, washing the membranous ghosts several times with 0.01 M Tris (pH 7.5).

Fragmentation of ghosts in crude lysates was done with sonic treatment in an 11-ke Raytheon oscillator for the times indicated in the text. The pieces which still sedimented in 20 min at 10,000 × g were then washed several times with buffers containing the Mg²⁺ concentration required.

Fization of sectioned material. Fractions were processed for electron microscopy either as suspensions or as lightly packed pellets of ghosts. Both the pellets and suspensions were doubly fixed. Material was first fixed with 2% glutaraldehyde (Sabatini, Bensch, and Barnett, 1963) buffered with 0.05 M phosphate (pH 7.2) for 4 hr; then, after an overnight wash in 0.1 M phosphate buffer, the samples were treated with 1% osmium tetroxide in phosphate buffer (Millonig, 1961) for 2 hr.

Dehydration of the fractions was carried out in a graded series of mixtures of ethyl alcohol and propylene oxide. They were then embedded in Epon (Luft, 1961) or an Epon-Araldite (Glauert and Glauert, 1958) mixture. Sections were cut on an LKB microtome and then mounted on carbon-coated or uncoated copper grids and examined in a Siemens Elmiskop I electron microscope. Sections were stained with either uranyl acetate (2% in 75% ethyl alcohol) or lead citrate (Reynolds, 1963), or with a combination of the two.

Negative staining of unfixed material. To permit observation of unfixed material, 1% uranyl acetate or 1% phosphotungstic acid was used. Uranyl acetate was dissolved in doubly distilled water and adjusted to pH 5.2 to 5.4 with sodium hydroxide immediately before use. Phosphotungstic acid was dissolved in distilled water and adjusted to pH 7.0 with either sodium hydroxide or potassium hydroxide. The most reproducible results were obtained when diluted portions of the material were mixed with stain and allowed to dry on a carbon-coated grid. The clearest preparations of unfixed ribosomal aggregates were obtained with the uranyl acetate stain, in agreement with the findings of Huxley and Zubay (1960).

Results

Amount of ribonucleoprotein bound to reticulum. Binding as a function of Mg²⁺ concentration was investigated. When cells were lysed and the membranes were washed in different concentrations of Mg²⁺, the amount of ribonucleoprotein bound to the membranes was a continuous function of the concentration of Mg²⁺ (Fig. 1; Schlessinger, 1963). In the absence of Mg²⁺, membranes contain essentially no ribonucleoprotein, whereas in 0.02 M Mg²⁺ they contained as much as 60% of the ribonucleoprotein in the lysate. The range of measurements was limited, because above 0.02 M Mg²⁺ the ribosomes began to form large aggregates (Tissières et al., 1959).

Electron micrographs of thin sections of ghosts usually show the cytoplasm only as a very dense mat of ribosomes within the limiting plasma membrane of the cell (Fig. 2). However, when sections were prepared from lysed protoplasts, roughly spherical particles approximately 150 to 200 Å across, which took up the uranyl stain for nucleic acids, could be distinguished. The number of ribosomes seen in sections of washed ghosts, like the total amount of RNA bound (Fig. 1), is lower the lower the concentration of Mg²⁺ (Fig. 3 to 5).

If the ribosomes were bound at the external limiting membrane of the ghost, they would be expected to cluster there. Instead, they were scattered throughout the interior of the ghost. They appeared to be spread out against a rather diffusely scattered reticulum, visible even in preparations which were not fixed with osmic acid (Fig. 6). Several earlier authors have observed ghosts under conditions (usually very low concentrations of Mg²⁺) in which ribosomes were reduced to a nearly negligible level (Fitz-James, 1964; Hendler et al., 1964), and a reticulum of similar appearance was described by Hendler et al. (1964), Abrams, Nielsen, and Thaemert (1964), and Pfister and Lundgren (1964).

We believe that the distribution of ribosomes in thin sections made in low Mg²⁺ concentrations, where few ribosomes remain bound, is comparable to the distribution in sections made in high Mg²⁺ concentrations, where many ribosomes are bound, for the following reasons. (i) The distribution of ribosomes remains fairly uniform at
Fig. 2a. Section of intact Bacillus megaterium cut in a transverse plane. The cytoplasm of the bacteria is filled with closely packed ribosomes. The pale zones are probably deoxyribonucleic acid, since comparable areas in protoplasts are empty after treatment with deoxyribonuclease. The cell membrane of each bacterium is surrounded by a cell wall which is completely removed by treatment with lysozyme (b). \( \times 120,000 \).

Fig. 2b. Section of a protoplast after treatment with lysozyme and deoxyribonuclease. The protoplasts appear larger than the intact bacteria, and their ribosomes are correspondingly less closely packed. \( \times 40,000 \).
FIG. 3 AND 4. Ribosome populations in protoplast ghosts subjected to media containing progressively decreasing concentrations of magnesium. The ribosomes in ghosts washed in $10^{-3} \text{M} \text{Mg}^{2+}$ still fill the cytoplasm of the cells (3a). When the concentration of $\text{Mg}^{2+}$ is lowered, ghosts contain progressively fewer ribosomes (3b, 4). $\times 100,000$. (Figure 4 is on p. 460.)
FIG. 4. See p. 459 for legend.

FIG. 5. In sections of ghosts washed in $10^{-3} \text{M Mg}^{2+}$, the remaining ribosomes appear to be suspended within the cell membrane. Ribosomes or polyribosomes seem to be attached to each other by or on a reticulum even after 5 min of sonic treatment. Although the cell membranes have been disrupted, the huge ribosomal aggregates persist. $\times 75,000$. 
each concentration of Mg\textsuperscript{2+}; there is no qualitative change observed in the photographs of sections as materials are prepared in progressively lower concentrations of Mg\textsuperscript{2+}. (ii) The amount of protein formed by these membrane-bound ribosomes per unit of RNA is about the same for fractions prepared in high or low Mg\textsuperscript{2+} concentrations, and is two to five times higher than the amount formed by free ribosomes (Schlessinger, 1963).

Thus, the use of graded concentrations of Mg\textsuperscript{2+} in the preparation of ghosts yields preparations which have progressively fewer polyribosomes remaining. These polyribosomes can now be seen displayed on a reticulum, which tends to remain positioned within the relatively intact plasma membrane (Fig. 3 to 6).

We did not expect the plasma membrane in low concentrations of Mg\textsuperscript{2+} to be as intact morphologically as it is (Fig. 4), since it had been reported that ghosts in low Mg\textsuperscript{2+} start to disappear (and presumably fragment; Weibull, 1953). Figure 1 shows, however, that the release of ribonucleoprotein from ghosts in low Mg\textsuperscript{2+} is accompanied by a very sharp drop in the refractility of the preparations. Thus, the ghosts do become translucent when studied with a light microscope.

When essentially all the Mg\textsuperscript{2+} is removed by EDTA treatment, the last ribosomes detach from the ghost, leaving only shreds of the reticulum very near the limiting membrane.

Because the holes made in the plasma membrane during osmotic lysis might not be large enough to permit polyribosomes to leave the ghost, the concentration of polyribosomes in ghosts could have been due to physical entrapment. We therefore performed the following controls.

Ultrasound is a powerful means of fragmenting cell membranes, particularly when the tough outer wall has been removed, as it is by the lysozyme treatment (Salton, 1957; Fig. 2). If ribosomes were simply trapped inside ghosts, they should be freed into the medium when membranes are sonically disrupted. Figure 5 shows, however, that after 5 min of sonic treatment, when ghosts have been fragmented, most of the ribosomes remain bound. Some ribosomes and polyribosomes do appear to be freed by this treatment, but orcinol tests and ultraviolet-absorption measurements indicated that 70% of the RNA still sediments with the remaining membranous debris after several further washings. When intact cells were sonically treated without any prior treat-

Fig. 6. Section of protoplast ghost after fixation in glutaraldehyde alone and staining with uranyl acetate. Without the nonspecific density contributed by osmium tetroxide, the ribosomes on the reticulum are the only structures defined by this relatively specific nucleic acid stain. × 120,000.
ment with lysozyme or deoxyribonuclease, the same amount of RNA continued to be bound to fragments of ghosts (unpublished data).

Increasing the time of sonic treatment (intervals from 30 sec to 30 min) resulted in a progressive breakdown of the massive ghosts, and also produced increasing numbers of single ribosomes and small cell fragments.

When such preparations were negatively stained with either uranyl acetate or phosphotungstic acid, large aggregates of ribosomes were clearly demonstrated (Fig. 7 and 8). Some aggregates were found still attached to fragments of the cell membrane, but many were also seen without any connection to remnants of the membrane (Fig. 8). Despite the increased resolution and clarity of structural detail provided by the negative-staining technique, the structural basis for the ribosomal aggregation is not clearly depicted. It is apparent, however, that this proposed reticular network which binds the ribosomes is not made up of the usual "unit membrane" which comprises the endoplasmic reticulum in mammalian cells (Palade and Siekevitz, 1956a, b; Sjöstrand, 1963).

Properties of the binding of ribosomes to reticulum. The binding of ribosomes in ghosts is Mg$^{2+}$-dependent, which suggests that the bonds are weak. However, once ribosomes were released in low Mg$^{2+}$ concentrations, they did not reassociate with membranes when the Mg$^{2+}$ concentration was raised again (unpublished data). If the ribosomes were adsorbed only weakly, then at any particular concentration of Mg$^{2+}$, ribosomes
bound to membranes might be expected to exchange with ribosomes in the surrounding medium. To test this possibility, portions of washed ghosts or sonic-treated fragments, bearing ribosomes labeled with $^{14}C$-uracil, were incubated in series of four tubes. Equal volumes of 0.01 M Tris, 0.01 M Mg$^{2+}$ containing 0, 1.5, 3-, or 6-fold the amount of ribosomes bound to the membranes were added to successive series, which were then incubated at 0, 20, or 30 C. At intervals, portions

**Fig. 8.** Fragments of ghosts treated as described in Fig. 7. Large ribosomal aggregates are seen which are not attached to pieces of plasma membrane from the original protoplasts. This appearance again suggests that whatever is binding the ribosomes together extends throughout the cytoplasm of the cells. Despite the greater resolution provided by the technique of negative staining, there is no recognizable membranous element linking the ribosomes together. X 180,000.
from samples of 15 observed that data susceptible component the change of added that 2,000 ug fraction for brane-bound at 20 C; 20% Mg count/min at result illustrated (Schlessinger, 1963) withdrawn then experimental points are scattered within the drawn bar. Upper line: 0.3 ml containing 330 ug of ghosts (O) as above, or ghosts and 1,000 ug of ribosomes (●) incubated at 20 C in the presence of 1 ug of ribonuclease. At the times shown, membranes were centrifuged out of portions from each tube; counts per minute were measured on 0.02-ml samples of the supernatant fluids. All were withdrawn from each tube and rapidly centrifuged. If progressive exchange of membrane-bound for unbound ribosomes were to occur, then increasing amounts of radioactivity would appear in the supernatant fluid. The results illustrated in Fig. 9 show that a constant fraction of the ribosomes was spontaneously released from the membranes, whether or not any additional ribosomes were added (10% of the total ribosomes at 20 C; 20% at 30 C). However, the bulk of the ribosomes did not exchange with externally added ribosomes, again suggesting that the binding is firm.

Ribonuclease treatment did not facilitate exchange of bound ribosomes (Fig. 9), indicating that the ribosomes are not bound by an RNA component susceptible to enzymatic attack. The data of Fig. 9 corroborate the earlier statement (Schlessinger, 1963) that treatment for 10 min at 20 C with 2 ug of ribonuclease per ml releases about 15% of the ribosomes bound to membranes. The fraction which remains bound can still be observed in a reticular pattern.

Similarly, treatment with deoxyribonuclease did not release bound ribosomes. All preparations are ordinarily treated with deoxyribonuclease (see Materials and Methods), but, even when washed ghosts were treated with 1 ug of deoxyribonuclease per ml for 30 min and then further washed, the ribosomes were still aggregated (Fig. 10).

We have examined further the earlier finding that about 60% of the RNA bound to ghosts is released by treatment for 10 min with 0.2% deoxycholate (Schlessinger, 1963). In electron micrographs of negatively stained material freed from either intact or sonically fragmented ghosts, small aggregates of ribosomes and many single ribosomes can be seen (Fig. 10).

**DISCUSSION**

Ribosomes bound to "microsomal membranes" in homogenates of mammalian tissues have been carefully correlated with ribosomes observed on endoplasmic reticulum in electron micrographs (Palade and Siekevitz, 1956a, b). A number of analogous observations have been made on the microsomal fraction in extracts of rat liver (Henshaw, Bojariski and Hiatt, 1963; Littlefield et al., 1975) and ribosomes bound to reticulum in the bacterial extracts (Schlessinger, 1963): (i) the bound ribosomes can be released by treatment with 0.2% sodium deoxycholate; (ii) the fraction of ribosomes bound is a function of the Mg2+ concentration; (iii) the ribosomes remain attached after repeated washes with buffer; (iv) the bound ribosomes are more competent in forming protein; and (v) the ribosomes seem to be released from reticulum when protein synthesis has slowed or stopped (e.g., after rats are starved for amino acids, after bacteria are treated with actinomycin D, or after cessation of protein synthesis in bacterial extracts).

Although ribosomes in bacteria and those in mammalian cells are similar in terms of the properties described above, they differ strikingly in their apparent organization within the respective cell types. Micrographs show no membranous structures in bacteria which correspond to the endoplasmic reticulum in mammalian cells; nor do most of the polyribosomes appear to be directly bound to the cytoplasmic membrane.

The possibility remains that polyribosomes exist free in intact bacteria; the cytoplasmic reticulum we observe would then be an artifact composed of material which denatures and binds polyribosomes at the time of osmotic lysis. However, ribosome function is promoted in ghosts, whereas it would very likely be disrupted or inhibited by a matrix of nonspecific, denatured
Fig. 10a. Treatment of sonically treated fragments of ghosts with additional deoxyribonuclease does not affect the size or the form of the ribosomal aggregates. $\times 120,000$.

Fig. 10b. Treatment with 0.2% deoxycholate releases most of the bound RNA, as described in the text, and also disrupts the largest aggregates of ribosomes. $\times 120,000$.

Material. Rather, the similarities detailed above suggest, therefore, that a reticulum exists in the cytoplasm of bacteria which, although not morphologically identical to the endoplasmic reticulum of mammalian cells, might have similar functional properties.
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LITERATURE CITED