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The Growth of Ordered Two-Dimensional Sheets of Ribosomal Particles from Salt–Alcohol Mixtures

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A procedure for the *in vitro* growth of well-ordered two-dimensional sheets from ribosomal particles using salts and salt–alcohol mixtures has been developed. Employing this procedure, ordered two-dimensional sheets of the wild type as well as of mutated 50 S ribosomal subunits from *Bacillus stearothermophilus* can readily be obtained. These sheets, stained with uranyl acetate or gold–thioglucose, are suitable for three-dimensional image reconstruction. They consist of relatively small unit cells with dimensions of 160 ± 15 and 365 ± 20 Å. Diffraction patterns of electron micrographs of these sheets contain features to 25 Å resolution.

KEY WORDS: crystallization; ribosomes; *Bacillus stearothermophilus;* two-dimensional sheets; vapor diffusion; diffraction pattern.

A better understanding of the molecular mechanism of protein biosynthesis depends on the availability of a reliable model for the ribosome. Diffraction techniques, namely X-ray crystallography and three-dimensional image reconstruction, are the most suitable methods for obtaining such a model. Along with crystallographic studies on intact ribosomal particles (1–3), we have developed two *in vitro* procedures, using alcohols (AL)[1] or salt–alcohol mixtures (ST), for the growth of two-dimensional sheets. Employing these procedures, ordered two-dimensional sheets of the wild type as well as of mutated 50 S ribosomal subunits from *Bacillus stearothermophilus* can readily be obtained (4,5).

One of the two procedures (AL), which is described in (4), is similar to that used for the growth of three-dimensional crystals (6). In this procedure, as for growing large three-dimensional crystals, the technique of vapor diffusion (7) has been employed, and volatile alcohols were used as precipitants. However, the relative concentrations of Mg^{2+} versus particles required for the production of the two-dimensional sheets were 50- to 100-fold higher than those needed for growth of three-dimensional crystals.

Because many technical difficulties are associated with structure determination of crystals which have been grown by volatile solvents, a large variety of solid or less volatile materials have been systematically examined for crystallization of intact ribosomal particles. Recently, we were able to develop a procedure for the growth of well-ordered two-dimensional sheets using salts and salt–alcohol mixtures as precipitants. The significance of this achievement stems not only from the possible use of these sheets for structural analysis, but also from the potential for obtaining three-dimensional crystals

[1] Abbreviations used: AL, procedure using alcohol; ST, procedure using salt–alcohol mixtures; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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of ribosomal particles from eubacteria using salts rather than alcohols. It should be mentioned that in parallel with the growth of the two-dimensional sheets from 50 S ribosomal subunits of eubacteria, large three-dimensional crystals of the same particles from the archaebacterium *Halobacterium marismortui* have been obtained using polyethylene glycol as a precipitant (3,8,9).

In this paper we describe the growth and the properties of two-dimensional sheets of 70-S ribosomes and of their 50-S subunits from *B. stearothermophilus* using salt–alcohol mixtures.

METHODS

Preparation of ribosomal particles. Cells of *B. stearothermophilus* (strains 799 and NCA-1503) were grown in 50-liter fermenters (10,11). The bacteria were harvested in the early log-phase by continuous flow centrifugation and stored at −80°C. Ribosomes were prepared by differential centrifugation after the cells were ground with aluminum powder. Ribosomal subunits were separated in Ti15 zonal rotors. The subunits were pelleted by high-speed centrifugation or by precipitation with 10% polyethylene glycol 6000 followed by low-speed centrifugation. It is crucial that the reaction mixture not be frozen at any stage of the preparation.

The integrity and the biological activity of the ribosomal particles were defined according to three criteria: (i) migration profiles in sucrose gradients obtained by centrifugation in a SW60 rotor; (ii) two-dimensional gel electrophoresis of ribosomal proteins using the procedures described in (12); (iii) activity test in a poly(U) system according to (13).

Production of two-dimensional sheets. To ribosomal particles (2–4 mg/ml) in H-I buffer (6), salts and methanol were added to final concentrations of 1.5 M $(NH_4)_2SO_4$, 0.15 M $MgCl_2$, 1 mM spermine, and 4% methanol. This solution had a pH of 6.2. Aliquots of 7–10 μl were applied to electron microscopy grids, left for 10 s, and washed with H-IM buffer (10 mM Hepes, pH 7.8, 90 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM β-mercaptoethanol). The specimens were stained for 10 s with either 0.5% (w/v) aqueous uranyl acetate or 1% (w/v) aqueous gold–thioglucose. Excess liquid was blotted off. The staining solutions were used without adjusting the pH.

Copper grids, 200 mesh, coated on the shiny side with 1% nitrocellulose-reinforced carbon films, were used. The crystallization solution was applied to the rough side of the grid. The grids were examined using a 400T Philips electron microscope, operating at 80 kV, at an electron optical magnification of ×17,000–96,000. Micrographs were checked by optical diffraction for focus and astigmatism correction. Optical diffractograms were used for the determination of unit cell dimensions and resolution limits.

RESULTS AND DISCUSSION

Although the production of the two-dimensional sheets is very reproducible, the quality and the size of the sheets depend, in a manner not yet fully characterized, on the bacterial strain. Thus, the exact conditions needed for the production of high-quality two-dimensional sheets have to be defined for each preparation.

Two-dimensional sheets also grow from solutions that do not contain alcohols. Addition of methanol to the crystallization mixture speeds up the production of the sheets, probably because it induces nucleation (14). To obtain better ordered two-dimensional sheets, advantage has been taken of the major role that the Mg^{2+} concentration plays in crystallization of the 50-S ribosomal subunit from bacterial sources (2,3). It was observed that three-dimensional crystals of 50-S ribosomal subunits from both *B. stearothermophilus* and *H. marismortui* grow in a relatively low Mg^{2+} concentration, whereas the growth of two-dimensional sheets from

B. stearothermophilus using alcohol as precipitating agent (4) requires Mg^{2+} concentrations that prohibit growth of three-dimensional crystals. Relatively high concentrations of Mg^{2+} ions are also needed to obtain large two-dimensional sheets from salts or salt–alcohol mixtures (ST type), since it was observed that Mg^{2+} could not be fully replaced by monovalent anions. Thus, in spite of the high concentration of $(NH_4)_2SO_4$ in the crystallization mixture, the Mg^{2+} concentration used in the (ST) procedure is 10- to 20-fold higher than that of the storage buffer or that used for the growth of three-dimensional crystals.

Because only a fraction of the particles in the crystallization medium comprises the two-dimensional sheets, these cannot be separated from the rest of the crystallization mixture. Evidence for the integrity of the ribosomal particles can be obtained by testing the biological activity and the migration profiles of the entire crystallization medium, or by examining the results of three-dimensional image reconstructions. We have shown that the biological activity and the migration profile of the ribosomal particles in the crystallization drop are not different from those of control particles not used for crystallization (11). Furthermore, the reconstructed images of both 50 and 70 S particles show well-defined shapes (15,16), and these results were obtained reproducibly in several reconstruction studies.

Since 50 S ribosomal particles are asymmetric objects, their structure cannot be internally averaged. Internal symmetry can contribute significantly to the interpretation of the structure of complex assemblies, as was shown, for instance, for spherical viruses (17–20). However, ribosomal particles may pack in plane or space groups which contain symmetry operations. In fact, most of the naturally produced sheets have fourfold symmetry and are composed of tetramers (21). Tetramers are also the basic units from which the *in vitro*-grown two-dimensional sheets from 50 S of *Escherichia coli* are built (22) and probably also of the three-dimensional crystals of the large ribosomal subunits from *B. stearothermophilus* (2). Lower symmetry, namely twofold, has been observed for the two-dimensional sheets from the large ribosomal particles from the same species, using alcohols as precipitants (4). Although it appears that the current salt-grown sheets also contain crystallographic symmetry, it was found that this holds only in low resolution.

The current two-dimensional sheets of the 50 S ribosomal subunits from *B. stearothermophilus* are suitable for three-dimensional image reconstruction. Such studies have already been carried out and have yielded a detailed model revealing a tunnel within the particle (15). The sheets were stained with uranyl acetate or gold–thioglucose. Regardless of the staining procedure, it was observed that they consist of relatively small unit cells with dimensions of 160 ± 15 and 365 ± 20 Å. They are well ordered, and the diffraction patterns of electron micrographs of sheets stained with gold–thioglucose contain features to 25 Å resolution. Although arrays stained by uranyl acetate usually seem to be larger and clearer than those stained with gold–thioglucose, the diffraction patterns of their micrographs extend to somewhat lower resolution. This might stem from the fact that uranyl acetate is not a pure negative stain and may interact with selected parts of the particle. The extent of this interaction depends on the accessibility of the different ribosomal components to the stain and therefore can be somewhat irregular.

Unfortunately, so far these sheets cannot be investigated unstained and frozen since the crystallization depends strongly on the nature of the grids. Those suitable for production of two-dimensional sheets break upon cooling. Attempts to overcome this problem as well as to improve the resolution of the stained sheets and to reconstruct a

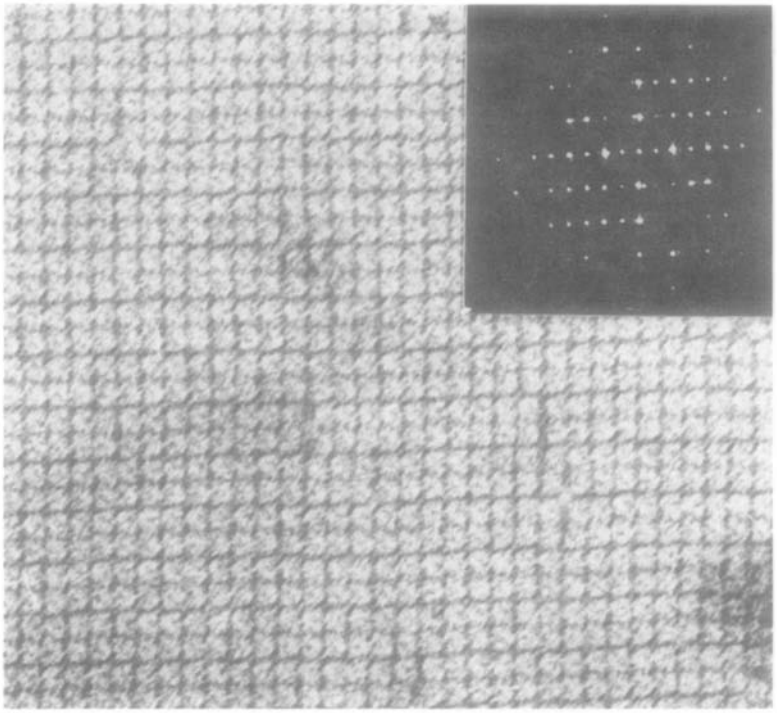

FIG. 1. An image of a two-dimensional sheet of *B. stearothermophilus* 70 S ribosomes (×28,000) stained with gold–thioglucose and an optical diffraction pattern from an area containing about 20 × 15 cells.

model from the currently available stained sheets are in progress.

Using the crystallization method described in this paper, ordered two-dimensional sheets have also been obtained from intact 70 S ribosomes of *B. stearothermophilus* [Fig. 1 and (23)] as well as from 50 S ribosomal subunits of a mutant lacking the ribosomal protein L11 (24).

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