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Three-dimensional crystals of ribosomal particles

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The central role of ribosomes in protein biosynthesis has stimulated extensive studies concerning their function and their biological, chemical, physical and genetic properties[1–4]. The results of these studies have illuminated many aspects of the process of protein biosynthesis but have also emphasized the crucial need for a molecular model for the understanding of its detailed mechanism. Thus, the time is now ripe to attempt three-dimensional structure analysis, using diffraction techniques.

Diffraction occurs as a result of the interaction of a beam with a periodically organized object with spacings comparable in magnitude to that of the beam wavelength. For structural studies the periodic objects are crystals which diffract the X-ray beam. The repeating element of a crystal, a parallelipiped shaped block, is the unit cell (Fig. 1).

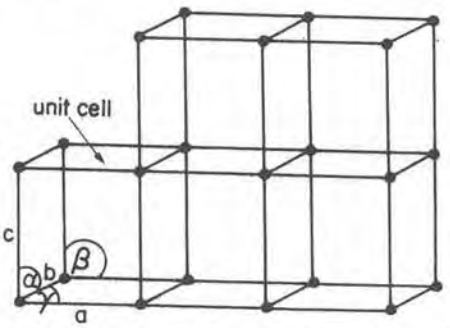

Fig. 1. A schematic representation of crystal packing.

Each unit cell is defined by three vectors (a,b,c) and three angles (α,β,γ). The crystals may contain symmetry elements (e.g. rotation axes). In these cases the unit cells contain two or more identical particles, which are related to each other by the symmetry operations of the crystal. Since diffraction may be considered in terms of reciprocal space, large unit cells give rise to diffraction patterns of small spacings, which in extreme cases are difficult to resolve. Although the effectiveness of crystallographic data collection has recently greatly advanced, the determination of the molecular structure of ribosomes is sure to require a high level of sophistication because of their large size, their instability and flexibility, and the complexity and asymmetry of their structure. Nevertheless such studies seem to be feasible and the expected results justify major efforts.

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Two-dimensional arrays of eukaryotic ribosomes

The first step in diffraction studies is crystal growth. For ribosomes, even this step seemed, until recently, to be a formidable task. The only source of encouragement was that, in spite of the unfavorable properties mentioned above, ribosomes of some eukaryotic species self-associate *in vivo* in ordered forms, under specific conditions. Periodic packings have been observed in *Entamoeba histolytica*[5], *Entamoeba invadens*[6], chick embryos[7–9], lizards[10–12] and the brains of senile humans[13]. Some of these forms have proved useful for low resolution structural studies:

Positively-stained sections of helices of the chromatoid body of *Entamoeba invadens*[6] gave rise to a reconstructed body consisting of three unequal regions. Membrane bound double layers of ribosomes from the oocytes of the lizard, *Lacerta sicula*, are formed during hibernation or after prolonged treatment with cold[11,12]. The ribosomes are organized as tetramers in a lattice with unit cell dimensions of $a = 59.5$ nm. Comparison of the three-dimensional reconstructed models obtained at 6–7 nm resolution from sheets contrasted with gold thioglucose and glucose shows that the rRNA chains are concentrated in a central core with few accessible sites on the surface, and the ribosomal proteins are located mainly at the periphery. The two subunits of the ribosome are partially resolved and the subunit interface is rich in rRNA. The large ribosomal subunits are located in the center of the tetramer and provide the inter-tetramer contacts. A similar arrangement has been observed in studies performed on sheets from which small subunits have been removed[11].

Tetramers of ribosomes have also been observed as building units of the ordered sheets formed *in vivo* of slowly chilled, early chick embryos. Two-dimensional arrays with a similar arrangement and unit cell dimensions of $a = 59.3$ nm have been produced *in vitro* with internal order of 6 nm, in which the asymmetric unit consists of a tetramer of whole ribosomes together with four non-ribosomal proteins[9]. X-ray diffraction patterns of partially-oriented gels of these sheets and three-dimensional reconstruction studies of negatively stained specimens have been used to obtain information about the internal packing within these arrays.

Hirano bodies, which are stacked sheets of membrane bound ribosomal particles, appear in the brains of senile humans[13]. Spatial Fourier filtering of stained sections of these tissues shows that the particles are packed in layers of a rhombic lattice with

$$a = 13 \text{ nm}, \alpha = 56°,$$

in which the average interparticle distance is 21.5 nm.

Three-dimensional crystals from bacterial sources

The natural capacity to form two-dimensional sheets has recently been extended to a higher degree of organization by the growth *in vitro* of three-dimensional crystals of bacterial ribosomes. These were chosen since they provide a crystal growth system which is independent of events *in vivo*; their ribosomes are well characterized biochemically[2–4], and can be prepared in large amounts.

Crystallization was achieved after systematic exploration of the parameters that control this process and the development of an experimental procedure for fine control of the contents of the crystallization medium[14]. Once the method had been developed, several three-dimensional crystal forms, two-dimensional sheets and helical organizations were obtained reproducibly. These have been formed by the whole ribosomes from *E. coli*[15] and by the large ribosomal subunits from *B. stearothermophilus*[16–18]. Electron micrographs of positively-stained, thin sections of embedded three-dimensional crystals indicate regular packing of particles of a size similar to that previously observed using different physical techniques[4]. Some of the crystal forms and sheets are suitable for further three-dimensional structure analysis. The choice of appropriate techniques for these studies has been dictated by the quality of the crystals.

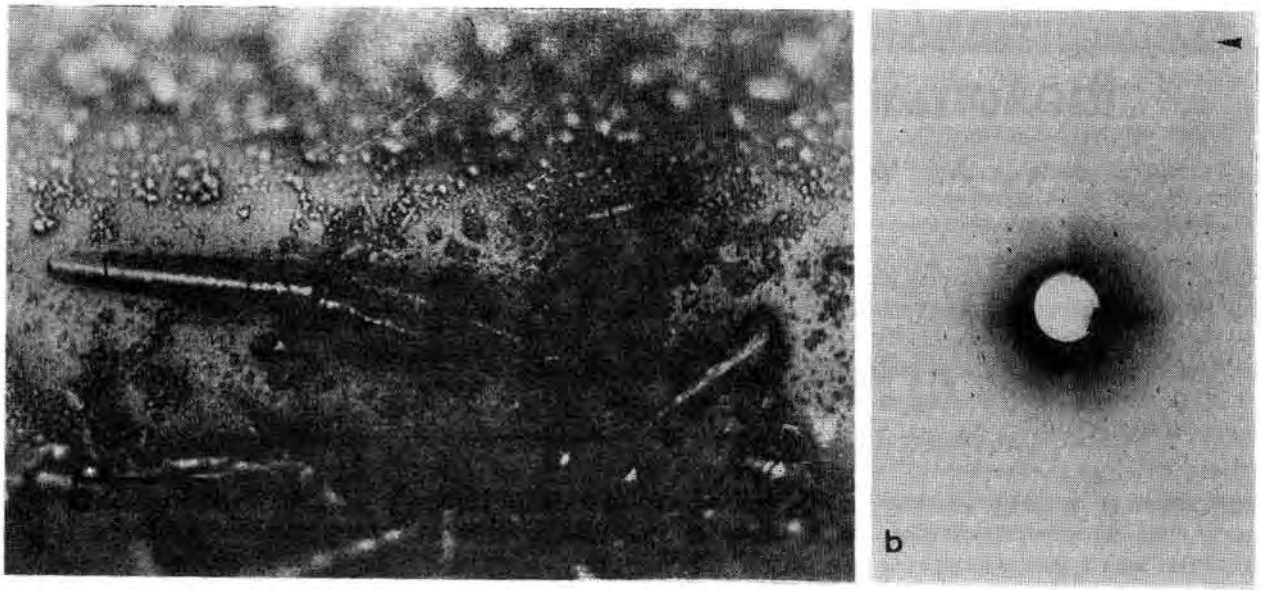

Fig. 2. (a) A typical crystal of the 50S ribosomal particles from B. stearothermophilus, *grown as described in Ref. 14 from a mixture of ethylene glycol and methanol at pH 8.4. In the early period of its growth the crystal was long, thin and intact. In later stages it became thicker and heavier, sank to the bottom of the crystallization drop and developed cracks perpendicular to its long axis. (b) X-ray diffraction pattern of 1(a) obtained in synchrotron beam (at the EMBL Outstation in DESY/Hamburg. The marked reflection is at about 1.8 nm. (The crystals were stable for more than three hours in the beam.)*

It is interesting that there is correlation between crystallizability and biological activity. Inactive ribosomal particles have not been crystallized. Moreover, in all cases, the *in vitro* crystalline material retains its biological activity, even for several months, in contrast to the short lifetime of isolated ribosomes in solution. This stability accords well with the hypothesis that when external conditions (e.g. hibernation) demand prolonged storage of potentially active ribosomes in living eukaryotic organisms, temporary periodic organization occurs *in vivo*.

X-ray diffraction studies

Three-dimensional crystals of the 50S particles from *B. stearothermophilus* have been used to get preliminary X-ray diffraction data[19]. Patterns have been obtained both from single crystals and from samples containing large amounts of microcrystals. The 'powder' patterns extend to 0.34 nm. They show fairly well-oriented, sharp rings, among them some with similar spacings to those previously reported for gels of ribosomes and extracted rRNA[20]. Such patterns may arise from partial orientation of the nucleic acid component within the particle.

X-ray patterns have been obtained from both native and crosslinked single crystals. In all cases the crystals were relatively stable in the X-ray beam (up to 3 h in synchrotron radiation), and their patterns contain resolved diffraction spots and indicate packing in relatively small unit cells, with dimensions similar to those obtained from image reconstruction studies of positively-stained sections of similar crystals (see below). Periodic spacings of 25.9 ± 0.3 nm and 13.9 ± 0.3 nm have been observed in patterns of crystals grown from methanol and 25.9 ± 0.2 nm, 20.7 ± 0.2 nm and 33.6 ± 0.2 nm in patterns of crystals grown from mixtures of methanol and ethylene glycol (Fig. 2).

There are some indications that the internal order of the crystals, as reflected by the resolution of their patterns (1.0–2.2 nm at 4–15°C), depends on the preparation as well as crystal growth conditions and is inversely correlated with their size, which can reach 0.9 × 0.25 × 0.15 mm. Since the crystals are grown from methanol and its mixtures, cooling to much lower temperatures is potentially possible and is expected to yield patterns of higher resolution. On the other hand, attempts to increase the size simultaneously with the order of the crystals by slowing down the crystallization process have so far failed, probably because of deterioration of the ribosomes before they were able to aggregate. It has been shown that under proper crystallization conditions, nucleation centers are formed within aggregates of ribosomal subunits during the initial period of crystallization[21]. However, significantly larger and fairly ordered crystals have already been obtained by changing the growth media from single to mixtures of organic materials (Fig. 2), and judging from the progress achieved, it is conceivable that soon even better crystals will be obtained.

Image reconstruction from electron micrographs of sectioned three-dimensional crystals

Three-dimensional crystals are too thick to be studied directly by electron microscopy. We used them to obtain information about their packing modes as well as a low resolution three-dimensional model of the stain distribution within the particles by image analysis of positively stained thin sections of the crystals[15]. The cell parameters have been determined for the crystals of the whole *E. coli* ribosome[15] and for five crystal forms of the large ribosomal subunit of *B. stearothermophilus* (Table I, and Refs 14, 16–18) using optical diffraction patterns of electron micrographs of sections in various directions. The cell dimensions are, in most cases, relatively small. Moreover, for two crystal forms (numbers 1 and 2 in Table I), they are in good accord with the periodic spacings determined from the X-ray patterns.

Three-dimensional image reconstruction studies were performed on a series of tilted, positively-stained (with uranyl acetate), thin sections of each of four crystal forms (numbers 1–4 in Table I) of the large ribosomal subunits from *B. stearothermophilus*[22]. All four forms show essentially the same distribution of stain within the particle. The model obtained from these studies is similar in size to that obtained by other physical techniques[4]. It consists of two domains of unevenly distributed density, which may result from preferential interactions of selected parts of the ribosomal sub-

Table I. Characterization of crystals of 50S ribosomal particles from B. stearothermophilus *using electron microscopy*

Crystal form	Cell parameters	γ (nm)
1[a]	13.0; 25.4	95°
2[a]	15.6; 28.8	97°
3	26.0; 28.8	104°
4	40.5; 40.5; 25.6	120°
5	21.3; 23.5; 31.5	127°

[a]May be related to each other.

unit with the stain. Comparison of this model with that obtained from sections stained with uranyl acetate together with phosphotungstic acid shows that the portion of the subunit that interacts with uranyl acetate (presumably the rRNA) is distributed mainly in the core of the particle, whereas the ribosomal components that interact with phosphotungstic acid (presumably the proteins) are located closer to the surface and are involved in interactions between the crystalline particles.

In vitro two-dimensional crystalline sheets of bacterial ribosomes

In spite of the valuable information obtained by three-dimensional structure determination from positively-stained sections, these studies are somewhat limited by the lower resolution of the sectioned material and by some difficulties in determining the exact direction of sectioning. Also, the interpretation of these images is hampered by uncertainty about the factors governing the stain distribution within the particle, the chemical nature of which is not completely defined.

The external contour of the particles may be obtained from negatively-stained, two-dimensional crystalline sheets. Thus, in parallel to the growth of three-dimensional crystals, there have been many attempts to produce sheets of bacterial ribosomes and their subunits. However, great difficulties have been encountered in the production of these sheets.

Small tubes and single layers of tetramers of the *E. coli* large ribosomal subunits have been obtained, with unit cell dimensions of $a = 33$ nm, $\alpha = 123°$[23]. Similar tetramers were also detected in the solution and it is conceivable that they provide the structural building unit for this form. Three-dimensional reconstruction studies at 4 nm resolution have been carried out for negatively-stained sheets, and the model obtained is reported to be consistent with the known features of this particle.

Two-dimensional crystalline sheets, which diffract to the 10th order (3 nm resolution) have been obtained from the large ribosomal subunit of *B. stearothermophilus* (Fig. 3 and Ref. 24). The method was essentially the same as that used to grow the three-dimensional crystals of the same material, but by varying the relative concentrations within the crystallizing medium and using active preparations of the 50S subunit that had failed to produce three-dimensional crystals detectable by light microscopy. It seems that the interparticle contacts which are created in the two-dimensional planes behave differently from those between planes in the three-dimensional crystals.

Each unit cell contains two particles and has relatively small dimensions (14.5 × 31.1 nm) which agree well with the forms of the three-dimensional crystals of the same 50S particles (Table I, Fig. 2 and Ref. 14). The small variations in cell constants among the different crystal forms may arise from the differences in the crystallization conditions as well as from the treatment in preparation for electron microscopy. Filtered images of the negatively stained sheets (Fig. 3c) show particles of similar dimensions to those obtained by other methods and with the characteristic features that have been detected by electron microscopy of single particles[4].

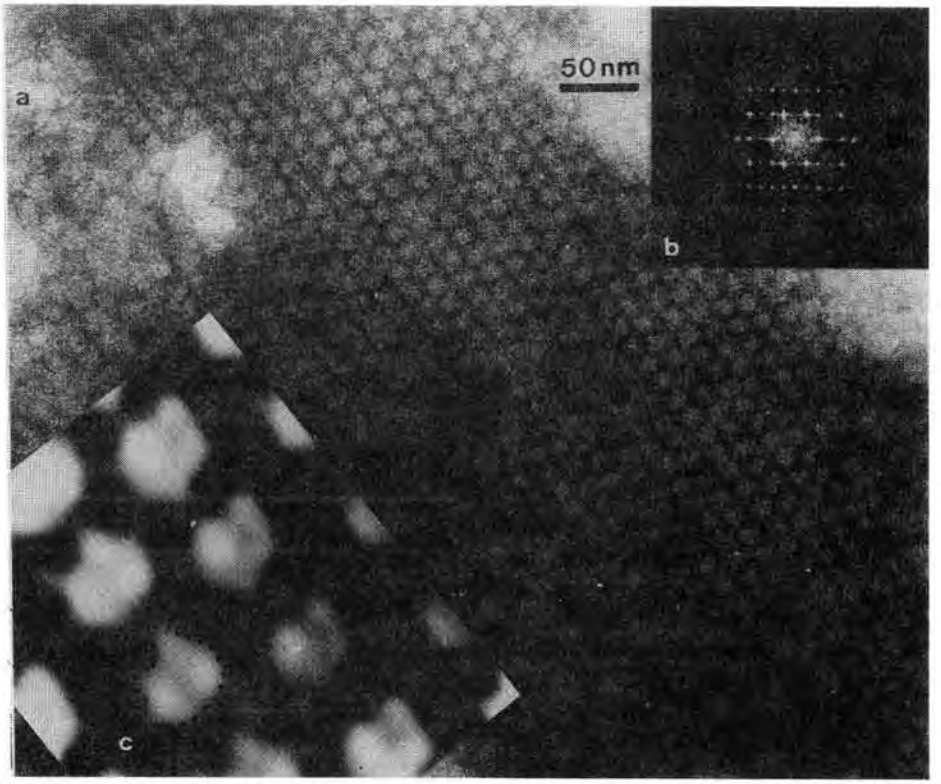

Fig. 3. (a) Electron micrograph of a negatively stained two-dimensional crystalline sheet of the large ribosomal subunit from B. stearothermophilus. *Grown as described in Ref. 24 from ethanol in the presence of 60 mM Mg^{2+}. (b) Optical diffraction pattern of 2(a). (c) Filtered image of 2(a). The outline of the particle is depicted.*

Future prospects

The ultimate goal of this line of research is to obtain a reliable three-dimensional model for the ribosome, which is essential for the understanding of its role in protein biosynthesis. The attractiveness of these studies, which arises mainly from their potentially great biological significance, is also conditioned by their expected feasibility, especially in view of the availability of suitable material, the reproducibility of crystal growth and the quality and the internal order of the crystals. Although the size of the crystals has still to be slightly improved to allow efficient diffraction studies, X-ray data collection from native crystals, using synchrotron radiation and maybe newly developed detectors, is not likely to be very difficult. Fortunately, the ribosomal particles are packed in crystals with relatively small unit cells, a reasonable degree of internal order and adequate stability in the X-ray beam.

Phases may be obtained by one or more of the following strategies: (1) From heavy-atom cluster derivatives, as used for structure determination of nucleosomes[25]; (2) Soaking the crystals, or co-crystallization of the 50S particles, with relatively small molecules which specifically bind to the 50S subunits and to which several heavy atoms can be

covalently attached; (3) Chemical modification of the ribosomes themselves – this might be achieved either by direct interaction of multi-metal-containing compounds with the intact ribosome prior to its crystallization or by reconstitution and crystallization of particles in which one or two proteins or the rRNA are loaded with heavy atoms; (4) Using information from neutron diffraction studies[26] or from image reconstruction studies of sections of the crystals as well as for two-dimensional sheets.

Since ribosomes from most bacteria disintegrate in high salt concentrations, crystallizations have been carried out from organic solvents. In order to overcome the inconvenience in handling such systems, alternative sources have been considered. One of these is *Halobacterium* from the Dead Sea, which contains ribosomes with the unusual property of withstanding and being active at high salt concentrations.

In spite of the exciting preliminary results, the way to detailed structure determination is still not completely paved and much effort is still required. However, there is already some light at the end of the tunnel, and the elucidation of the molecular conformation of ribosomes is expected to be achieved in the foreseeable future.

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