Crystallographic and Image Reconstruction Studies on Ribosomal Particles from Bacterial Sources

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Introduction

Diffraction methods are the most powerful techniques for reliable elucidation of molecular structures. Such structural information is essential for detailed understanding of mechanisms of biological processes, including protein biosynthesis. Application of diffraction techniques is dependent on the availability of crystalline material. Because of the enormous size, the instability and flexibility of ribosomes, and the intricate and asymmetric nature of their structure, the *in vitro* growth of three- and two-dimensional crystals seemed, until recently, to be a formidable task. Nevertheless, procedures for crystallization of intact ribosomal particles have been developed recently, and structural analysis of several systems is currently being performed.

In this chapter we discuss two techniques: X-ray crystallography and three-dimensional image reconstruction. Progress in structural studies of particles as large and as complex as ribosomes hinges on the correlation of the crystallographic data with electron microscopy. The large size of ribosomal particles, which is an obstacle for crystallographic studies, permits direct investigation by electron microscopy. Thus, electron microscopy can provide a useful tool for rapid evaluation and refinement of crystallization conditions. Using electron microscopy, the initial steps of crystallization can be detected and the tendency of native and modified particles to crystallize can be followed rather quickly, in contrast to the long time needed for the growth of large three-dimensional crystals. Results from electron microscopy can also be used to locate and orient the particles within the crystals and models obtained by three-dimensional image reconstruction may facilitate extraction of phase information. Thus, structure determination by three-dimensional image reconstruction from two-dimensional sheets is justified not only in its own right, but also because of its expected contribution to the determination of phases needed for crystallographic analysis.

Ribosomes from several eukaryotic species may, under special conditions, organize *in vivo* into two-dimensional sheets. Therefore they seem to be suitable objects for *in vitro* crystallization. On the other hand, ribosomes from prokaryotes provide a system for crystallization which is independent of *in vivo* events. They are smaller and have been characterized biochemi-
cally in much greater detail than those from eukaryotes. In addition, they can be produced in high purity and large quantity.

For effective crystallographic studies, a constant supply of crystallizable material is essential. Suitable sources are those from which one can obtain reproducibly high-quality ribosomes in sufficient amounts for refinement of the crystallization conditions as well as for the production of large crystals needed for data collection. It was found that for the crystallization particles, virtually all preparations of active material yield crystals or two-dimensional sheets. These include the whole 70S ribosomes of Bacillus stearothermophilus and E. coli, the 30S subunits of Thermus thermophilus and E. coli, and 50S subunits of B. stearothermophilus and Halobacterium marismortui. However, because of the intricate nature of the particles, the exact conditions for the growth of well-ordered large crystals still must be varied for each ribosomal preparation. Some details of the preparation are given below.

Cells of B. stearothermophilus (strains 799 and NCA-1503) are grown in 50-liter fermenters and those of H. marismortui in fermenters of 100 liters. The bacteria are harvested in early log phase by continuous flow centrifugation and stored at $-80^\circ$. Ribosomes are prepared by differential centrifugation after grinding the cells with alumina powder. Ribosomal subunits are separated in a Ti15 zonal rotor. The subunits of B. stearothermophilus are pelleted either by high-speed centrifugation or by precipitation with 10% polyethylene glycol followed by low-speed centrifugation, whereas those of H. marismortui are concentrated by ultracentrifugation. It is crucial that the preparation not be frozen at any stage of preparation.

The integrity and the biological activity of the ribosomal particles are defined by three criteria: (1) migration profiles in sucrose gradients, obtained by centrifugation in a SW60 rotor; (2) two-dimensional gel electrophoresis of ribosomal proteins using the procedures for B. stearothermophilus and H. marismortui, respectively; (3) activity in protein biosynthesis.

using the poly(U) system according to Nierhaus and co-workers\textsuperscript{8,9} for \textit{B. stearothermophilus} and \textit{H. marismortui}, respectively.

Three-Dimensional Image Reconstruction

Electron microscopy enables direct imaging of biological structures at a macromolecular level. Image reconstruction permits determination of three-dimensional structures from periodically ordered arrays. Combined with electron microscopy, image reconstruction is useful for determination of structures of large biological macromolecules and assemblies at moderate resolution. Three-dimensional image reconstruction has been developed recently, has grown in popularity, and is now considered a standard procedure. It involves averaging, by Fourier transformation, of images obtained by electron microscopy of tilt series of periodically organized identical objects. The principles of this method have been described.\textsuperscript{10}

There are several limitations to three-dimensional image reconstruction and to visualization of single particles by electron microscopy. These arise from the difficulties of preserving biological specimens in the microscope vacuum, from radiation damage, and from the influence of the staining procedure on the resulting model. However, there is a fundamental difference between structural analysis by electron microscopy and by three-dimensional image reconstruction. Whereas visualization of isolated particles is rather subjective, three-dimensional image reconstruction is based on diffraction and thus is inherently of a more objective character. Furthermore, it is conceivable that isolated particles tend to lie on grids in a few preferred orientations. As a result of the contact of the particles with the flat grids, their projected views are likely to be somewhat distorted. In contrast, particles within the crystalline sheets are held together by inter-particle contacts. These contacts construct a network which may stabilize the conformation of the particles and decrease, or even eliminate, the influence of the flat surfaces of the grids.

The advantages of three-dimensional image reconstruction from ordered two-dimensional sheets can be demonstrated in the cases of the 80S ribosomes from lizards\textsuperscript{11} and the 50S ribosomal subunits from \textit{B. stearothermophilus}.\textsuperscript{12} For both, the reconstructed models are of the same size and contain the features observed by visualization or reconstruction of

single particles by electron microscopy, but at the same time show key features (e.g., a long tunnel) which could not be detected otherwise.

The subjects for three-dimensional image reconstruction are either two-dimensional sheets or thin sections of embedded three-dimensional crystals. These are stained with the same materials used for conventional electron microscopy. For the elucidation of the external contour of particles, two-dimensional sheets are studied mainly unstained or negatively stained with inert materials. In contrast, thin sections of embedded crystals must be positively stained and, when reconstructed, yield information about the distribution of the stain within the studied object. Still, there are boundary cases. Not all negative stains are truly inert. Some, such as uranyl acetate, interact with selected compounds, e.g., rRNA, of the particles. On the other hand, positive stains, which should reveal the internal distribution of the material with which they interact, may contrast preferentially selected parts of the ribosomal particles according to the extent of their accessibility.

Reconstruction from unstained specimens is best since it is free from the stain influence, thus giving rise to models whose boundaries are determined by differences in contrast. Furthermore, such studies may show the internal distribution of various compounds of the particles. In addition, the diffraction patterns of unstained specimens usually extend to higher resolution than the comparable stained ones. Unfortunately, this procedure cannot be applied to two-dimensional sheets which have been grown in the presence of salts, since on cooling the excess salt crystallizes on the grids and prevents visualization of the two-dimensional sheets.

In favorable cases the influence of the staining procedure is minimal. One example may be the tunnel of the large ribosomal subunits. It is clearly resolved in all reconstructions of salt-grown sheets of the 50S subunits of *B. stearothermophilus*, independent of the staining material, as well as in the reconstructed model of the unstained sheets from chick embryos.

Three-dimensional image reconstruction has been successfully applied to some interesting biological systems including two-dimensional sheets and thin sections of crystals of ribosomal particles. However, because

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the order of the two-dimensional sheets is expressed only in a plane, because the objects are viewed in projection rather than in three dimensions, and because the data obtained by tilting are limited (data above a certain tilt angle, the "missing cone," and the (001) reflections are always absent), this technique alone is bound to provide only partial structural information.

In Vivo Grown Two-Dimensional Sheets of Ribosomal Particles from Eukaryotes

This article focuses on ribosomal particles from prokaryotes. Because the first objects to be studied by three-dimensional image reconstruction were naturally occurring two-dimensional sheets from eukaryotic ribosomes, a brief description of results obtained from these systems is given below. Under special stressful conditions (such as cooling, lack of oxygen, and hibernation) ribosomes of some eukaryotic species (lizard, chicken, amoeba, and human) associate with each other in vivo to form periodic objects such as helices and two-dimensional ordered layers.\textsuperscript{11,13-17,19-23} Furthermore, a semi-in vitro procedure for crystallization of ribosomes from chick embryos subjected to cold treatment has been developed in cell suspensions.\textsuperscript{15,24}

These sheets and helices are usually made of whole ribosomes and consist of relatively large unit cells. Furthermore, the ribosomes which comprise these sheets are bound to membranes, which may introduce noise when investigated by electron microscopy. In spite of these unfavorable properties, and because until recently there were no other systems of periodically packed ribosomal particles, they have been subjected to three-dimensional image reconstruction studies and have yielded useful low-resolution (55–120 Å) information. Sizes of several ribosomal particles have been determined, either directly from the reconstructed particle or indirectly from the unit cell parameters. Thus, the minimum size of the large subunit in brains of senile humans can be derived from lattices which consist of unit cells as small as 130 × 130 Å.\textsuperscript{19} Information was also derived about the distribution of materials within the particles. It was observed that the rRNA-rich regions are concentrated in the interior of the ribosomes as well as in the interface area between the large and small subunits. For lizard ribosomes a narrow elongated (150–200 Å length) region of low density has been detected. This region originates near the

\textsuperscript{20} B. Byers, \textit{J. Mol. Biol.} 26, 155 (1967).
subunit interface and passes through the rRNA-rich core to a point close to the membrane attachment site. It was assigned to be a channel which may provide a path for the nascent polypeptide.11

Tetramers are the building units of membrane-bound double layers of whole ribosomes from oocytes of the lizard Lacerta sicula as well as in the ordered sheets of early chick embryos formed in vivo and in cell suspensions.11,14,17 In both systems, the large ribosomal subunits are located in the center of the tetramer and are involved in the contacts within and between tetramers.

In Vitro Growth of Two-Dimensional Sheets of Ribosomal Particles from Prokaryotes

Most of the two-dimensional sheets from prokaryotic ribosomal subunits have been grown in vitro from low-molecular-weight alcohols by vapor diffusion in hanging drops.25-28 Recently, mixtures of salts and alcohols have been used for the growth of two-dimensional sheets in depression slides or on electron microscopy grids.29-31 Because only a small fraction of the particles in the crystallization medium comprises the two-dimensional sheets, these cannot be separated from the rest of the crystallization mixture. Thus, evidence for the integrity of the ribosomal particles in the sheets may be obtained indirectly by testing the biological activity and the migration profile of the entire crystallization medium.

The reconstructed model of the 50S particles from Bacillus stearothermophilus at 30 Å resolution12 shows substantially more detail (Figs. 1 and 2). It includes several projecting arms which are arranged radially near the presumed interface with the 30S subunit, around a cleft which turns into a Y-shaped tunnel of up to 25 Å in diameter, in which the longest distance is 100–120 Å. This tunnel spans the particle and may provide the path taken

32 Deleted in proof.
by the nascent polypeptide chain. When only the resolution features of up to 55 Å are included in the reconstructions of the 50S particles, the overall shape of the 50S particle is almost spherical and it contains only two thick, short arms. In this respect it resembles the models derived from visualization of isolated particles.

A tunnel in a similar location was also detected in the reconstructions of the whole 70S ribosome for *B. stearothermophilus* at 47 Å resolution. Other interesting details which were revealed by the three-dimensional reconstruction of the 70S ribosomes include an empty space in the interface of the two subunits large enough to accommodate the components of protein biosynthesis (e.g., tRNA and elongation factors), as well as a groove on the small subunit, rich in RNA, which may be the binding site for the mRNA.

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Thin Sections of Embedded Three-Dimensional Crystals

When no two-dimensional sheets or large three-dimensional crystals are available, three-dimensional microcrystals may be used for obtaining some three-dimensional information. Since microcrystals are too thick for direct investigation by electron microscopy, they may be sectioned into thin sections. These are positively stained and used for image reconstruction studies. The information obtained from such analysis is somewhat
limited by the lower resolution of the sectioned material and by difficulties in determining the exact sectioning direction. Furthermore, the interpretation of the images is hampered by uncertainty as to the factors governing the stain distribution within the particle, whose chemical nature is not completely defined.

In spite of these unfavorable factors, some valuable information concerning the internal distribution of the ribosomal components has been obtained from three-dimensional image reconstruction of thin sections of embedded three-dimensional crystals. Furthermore, in principle, the reconstructed images of the positively stained portions of particles may be incorporated within the outer boundaries obtained from three-dimensional image reconstruction of two-dimensional sheets.

Three-dimensional image reconstruction studies on positively stained thin sections of three-dimensional crystals of the large ribosomal subunits from *B. stearothermophilus* indicate that most of the ribosomal RNA is located in two domains in the core of the particle, whereas the proteins are located closer to the surface. This is in agreement with results obtained from unstained two-dimensional sheets of ribosomes from chick embryos and *Lacerta sicula*.

**Crystallography of Ribosomal Particles**

X-Ray crystallography is the only direct method for the determination of complete three-dimensional structures. Inherently, crystallographic studies are not affected by the limitations imposed by other diffraction techniques. About 30–60% of the volume of three-dimensional crystals of biological macromolecules is occupied by solution. Moreover, in contrast to the need to evacuate samples for investigation by electron microscopy, during crystallographic data collection the crystals are kept at high relative humidity, conditions under which a native conformation is likely to be preserved. In addition, the objects studied by X-ray crystallography are periodically arranged in three dimensions, whereas three-dimensional image reconstruction is performed on two-dimensional sheets which lie on flat grids in a specific manner. The geometrical properties of measuring devices—X-ray cameras, detectors, and diffractometers—used for crystallographic studies permit rotations in space. Hence, the three-dimensional crystals are investigated throughout the whole sphere of reflection rather than from a limited tilt series used for image reconstruction.

X-Ray structure determination of macromolecules has recently advanced rapidly, especially in the effectiveness in collecting, processing, and analyzing crystallographic data. Consequently, the structure of several
large biomolecular assemblies have been determined. However, full crystallographic structure analysis is still a lengthy stepwise procedure, requiring a high level of sophistication, much effort, resources, and advanced technology.

A description of the basic concepts of structure determination of biological macromolecules by X-ray crystallography is beyond the scope of this chapter. The reader is advised to consult several excellent books. Here we discuss the potential application of X-ray crystallography to structural determination of ribosomal particles.

Crystal Growth

Three-dimensional crystal of intact ribosomal particles were grown only after an intensive systematic exploration of crystallization conditions and the development of an innovative experimental procedure for fine control of the content and volume of the crystallization drop. Once the crystallization method was developed, it was found to be reproducible and crystals of 70S ribosomes from *E. coli*, 30S ribosomal subunits from *Thermus thermophilus*, as well as of 50S ribosomal subunits from *B. stearothermophilus* and from *H. marismortui* have been obtained. For each crystal form the quality of the crystals depends, in a manner not yet fully characterized, on the procedure used in preparation of the ribosomal subunits and on the bacterial strain.

It was observed that there is a correlation between crystallizability and biological activity. So far, inactive ribosomal particles could not be crystallized. Moreover, in spite of the natural tendency of ribosomes to disintegrate, all crystallized particles retain their biological activity, even for several months, in contrast to the short life time of isolated ribosomes in solution. This property accords well with the hypothesis that, when external conditions (e.g., hibernation) demand prolonged storage of potentially active ribosomes in living organisms, temporary periodic organization occurs in vivo.\textsuperscript{24,56}

All three-dimensional crystals from intact ribosomal particles have been grown by a common technique used in protein crystallography, namely vapor diffusion.\textsuperscript{57,58} It is based on a slow increase in the concentration of the crystallizing material (protein or nucleic acid) by controlled evaporation of the solvent in the crystallization drop. This is achieved in closed systems by equilibrating small droplets of the crystallizing solution, which also contains a precipitant, with reservoirs of solutions of higher concentration of the precipitant. For the crystallization of ribosomal particles from eubacteria this procedure was somewhat modified. Because these particles fall apart at high salt concentrations, volatile organic solvents were used as precipitants. The crystallization droplets, which contain no precipitant or an extremely small quantity of it, are equilibrated with a reservoir containing the precipitant as well as an inert salt. In this way, crystallization is facilitated by the diffusion of the organic solvent into the

\textsuperscript{57} D. R. Davies and D. M. Segal, this series, Vol. 22, p. 266.
drop simultaneously with evaporation of water from it.\(^5^9\) Two different crystallization systems were assembled: (1) hanging drops on glass plates, used mainly for survey since it leads to the growth of microcrystals, and (2) liquid columns in X-ray capillaries (Fig. 3), in which large crystals are obtained. Growing crystals from alcohols imposes many technical difficulties in handling, data collection, and heavy-atom derivatization. This is one reason for using ribosomal particles from \textit{Halobacterium}. These parti-

icles are stable and active at high salt concentrations and provide a system for crystallization from salts or less volatile compounds.\textsuperscript{5,55}

For ribosomal particles, as for many other biological systems, it was observed that there is an inverse correlation between the rate of crystal growth and the internal order of the crystals. This correlation is especially pronounced for crystals from halophilic ribosomes, since these grow very quickly, occasionally within a few hours. Attempts to increase the size of the crystals while enhancing their internal order by slowing down the crystallization process have been successful for the halophilic ribosomes. Whereas at very high salt concentrations only disordered microcrystals could be grown, larger and better ordered crystals were obtained as a result of a drastic reduction in the concentration of KCl in the crystallization mixture as well as in the reservoir (Fig. 4).\textsuperscript{31,54,55} However, application of similar procedures to \textit{B. stearothermophilus} has so far failed, probably because ribosomes from this source are less stable, and, upon slowing down the crystallization process, they deteriorate before they are able to aggregate and form proper nucleation centers.\textsuperscript{59}

Seeding is a procedure in protein crystallography for increasing the size of crystals. It is an extremely delicate procedure but, at the same time, rewarding. The crystal chosen for seeding is harvested from the crystallization mixture in a solution with slightly higher concentration of the precipitant and washed twice in this solution to dissolve micronucleation sites. After removing the washing solution, the crystal is transferred to a drop with a fresh crystallization mixture which has already been equilibrated with the proper reservoir.

Application of seeding technique was not possible for the crystals grown from the 50S subunits of \textit{B. stearothermophilus}. In contrast, this was the ultimate way for obtaining ordered crystals from the same particles from \textit{H. marismortui}. Advantage has been taken of the major role played by the Mg\textsuperscript{2+} concentration in crystallization of ribosomal particles. It was found that three-dimensional crystals of 50S ribosomal subunits from \textit{B. stearothermophilus} grow in relatively low Mg\textsuperscript{2+} concentration, whereas the production of two-dimensional sheets requires a high Mg\textsuperscript{2+} concentration, at which growth of three-dimensional crystals is prohibited. Similarly, for spontaneous crystal growth of 50S subunits from \textit{H. marismortui}, the lower the Mg\textsuperscript{2+} concentration is, the thicker the crystals are. With these points in mind, a variation of the standard seeding procedure has been developed. Thin crystals of the 50S subunits from \textit{H. marismortui} grown spontaneously under the lowest possible Mg\textsuperscript{2+} concentration are transferred to mixtures in which the Mg\textsuperscript{2+} concentration is so low that the transferred crystals dissolve, but after several days new microcrystals can be observed. These reach their maximum size after 3–4 weeks, are very well ordered, and 10- to 30-fold thicker than the original seeds (Fig. 4).
FIG. 4. Growth of large, ordered three-dimensional crystals of the 50S ribosomal subunits from *H. marismortui* by vapor diffusion at 19°. (Bar length = 0.2 mm.) (a) Microcrystals obtained within 1–2 days from 7–8% polyethylene glycol, in the presence of 2.5 *M* KCl,
The process of crystal growth is initiated by nucleation. Although many biological molecules and complexes have been crystallized, little is known about the mechanism of nucleation. Theoretical models have been developed for the nucleation of crystals of small molecules. However, most of the data currently available concerning the process of nucleation of crystals of biological systems are based on rather indirect evidence, such as monitoring aggregation under crystallization conditions by scattering techniques. Crystals of ribosomal particles provided an excellent system for direct investigation of nucleation. In this study, the crystallization process was interrupted before the formation of mature crystals, and the crystallization medium was examined by electron microscopy. It was found that the first step in crystal growth is nonspecific aggregation and that nucleation starts by a rearrangement within the aggregates.

A severe limitation in crystallographic data collection is the shape of the crystals. So far, all crystals of ribosomal particles have one very thin dimension which corresponds to the longest unit cell edge (see below). They grow either as thin needles (50S particles from *B. stearothermophilus*) or thin plates (same particles from *H. marismortui*). It is conceivable that, under microgravity conditions, well-ordered crystals with isotropic dimen-

0.5 M NH₄Cl, 0.15–0.20 M MgCl₂, and 10 mM spermidine in the crystallization mixture (pH 5.0–5.2), and equilibrated against 3.0 M KCl, 9% polyethylene glycol, 0.5 M NH₄Cl, and 0.20 M MgCl₂. (b) Crystals obtained within 2–3 days in droplets containing a lower KCl concentration than used in (a). The droplet of 4–5% polyethylene glycol, in the presence of 1.2–1.7 M KCl, 0.5 M NH₄Cl, 0.10 M MgCl₂, and 0.10 M MgCl₂. Insert: An X-ray diffraction pattern taken perpendicular to the thin axis of the crystals, obtained under conditions described in Fig. 6. (c) Crystals obtained within 3–5 days from droplets similar to those used for (b), equilibrated with reservoirs of lower KCl concentrations. The droplet of 4–5% polyethylene glycol, in the presence of 1.2 M KCl, 0.5 M NH₄Cl, 0.05–0.10 M MgCl₂, and 10 mM spermidine in the crystallization mixture (pH 5.0–5.6), was equilibrated against 1.7 M KCl, 9% polyethylene glycol, 0.5 M NH₄Cl, and 0.10 M MgCl₂. Insert: An X-ray diffraction pattern taken perpendicular to the thin axis of the crystals, obtained under conditions described in Fig. 6. (d) Crystals obtained by seeding of crystals from (c) in a crystallization drop containing 5% polyethylene glycol, 1.2 M KCl, 0.5 M NH₄Cl, and 0.03 M MgCl₂, at pH 5.6, which was equilibrated with 7% polyethylene glycol, 1.7 M KCl, 0.5 M NH₄Cl, and 0.03 M MgCl₂, pH 5.6. Seeds were small, well-shaped crystals, transferred in a stabilization solution of 7% polyethylene glycol in 1.7 M KCl, 0.5 M NH₄Cl, and 0.05 M MgCl₂, at pH 5.6. Insert: An X-ray diffraction pattern taken perpendicular to the thin axis of the crystals, obtained under conditions described in Fig. 6.

sions will be obtained. Thus, attempts to grow crystals of ribosomal particles in a space shuttle are currently underway.

Progress of Crystallographic Analysis

Synchrotron radiation provides the most intense, intrinsically parallel X-ray beam. Thus it permits diffraction patterns to be obtained with short exposures and allows beams of arbitrary size by use of slits. Synchrotron radiation is essential for crystallographic data collection from crystals of ribosomal particles. This is due to the large unit-cell dimensions (Table I), the shape of the crystals (see above), their fragility, and their relatively short life times. Using synchrotron radiation, the unit-cell parameters of three crystal systems have been determined and interpretable data have been collected. Thus, crystallographic studies are currently being performed on crystals from the small ribosomal subunits from *Thermus thermophilus* as well as from the large ribosomal subunits from *B. stearothermophilus* and *H. marismortui*.

It was observed that between -20° and 4° all crystal forms are stable in the synchrotron X-ray beam for a few hours. However, the reflections with resolution better than 20 Å decay within a few minutes of irradiation. This imposed serious experimental constraints. To eliminate crystal damage, crystals were aligned only visually and each of them produced only 1 or 2 rotation photographs. Thus, more than 260 crystals (of 50S subunits from *H. marismortui*) were used in order to collect a presumably full set of data.

In contrast, crystals which were immersed in viscous solutions, mounted on tiny glass rods or spatulas, flashed cooled, and irradiated at cryotemperature (around -180°) show hardly any radiation damage even after hours in the synchrotron X-ray beam. Thus complete data sets could be collected from single crystals. (A. Yonath, H. Hope, K. von Buehrlen, F. Frolov, C. Kratky, I. Makowski, and H. G. Wittmann, manuscript in preparation.)

Initially, crystals from the 50S subunits of *B. stearothermophilus* were obtained directly in X-ray capillaries by vapor diffusion of 4° from mixtures of methanol and ethylene glycol as long pointed needles which may reach the size of 1.5 × 0.3 × 0.2 mm (Fig. 5). Since most of them grew with one of their faces adhering to the walls of the capillaries, it was possible to irradiate them without removing the original growth solution. This was essential since any handling of these crystals is virtually impossible. Although most of the crystals grew with their long axes parallel to the capillary axis, a fair number grew in different directions. Thus, it was possible to determine the unit-cell constants (Table I) and to obtain dif-
<table>
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<tr>
<th>Source</th>
<th>Crystal form&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cell dimensions (Å) determined by</th>
<th>Resolution</th>
<th>Comments&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td><strong>70S E. coli</strong></td>
<td>A</td>
<td>340 × 340 × 590; 120°; P6&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>310 × 350; 105°</td>
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<td></td>
<td>2, P</td>
<td>148 × 186; 95°</td>
<td>13 Å</td>
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<td>3&lt;sup&gt;c&lt;/sup&gt;, P</td>
<td>170 × 180; 75°</td>
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<td>3, A</td>
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<td>4, A</td>
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<td>5, A</td>
<td>213 × 235 × 315; 120°</td>
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<td>6&lt;sup&gt;cd&lt;/sup&gt;, A</td>
<td>330 × 670 × 850; 90°</td>
<td>360 × 680 × 920; P2&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>285 × 380 × 526; C222</td>
<td>11 Å</td>
<td>D</td>
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<td>148 × 360; 109°; P1</td>
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<td>25 Å</td>
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<sup>a</sup> All forms are of three-dimensional crystals, unless marked by 2D. Crystals were grown by vapor diffusion from low-molecular-weight alcohols (A), MPD (M), polyethylene glycol (P), or ammonium sulfate (AS).

<sup>b</sup> D, crystallographic data have been collected from native and derivated (H) crystals.

<sup>c</sup> Same form and parameters for crystals of a complex of 50S subunits + tRNA + a segment (18–20mers) of a nascent polypeptide chain.

<sup>d</sup> Same form and parameters for crystals of large ribosomal subunits of a mutant (−L11 protein) of the same source.
Fig. 5. (a) Crystals of the 50S ribosomal subunits from *B. stearothermophilus* grown in 0.5 mm X-ray capillaries by vapor diffusion at 4°. Bar length = 0.2 mm. Crystallization mixture of 20 μl 50S ribosomal subunits (10–20 mg/ml) in H-I buffer, 10 mM spermine, 1% methanol, 10 mM HEPES or glycine buffer, pH 8.4, was equilibrated with a reservoir of 12% methanol, 12% ethylenediol, 0.5 M NaCl, pH 8.4. (b) X-Ray diffraction patterns from crystals similar to those shown in (a), obtained at −4° with synchrotron radiation (A1 station at CHESS/CORNELL University operating at 5 GeV, current 30–40 mA) with 0.3 mm collimated X-ray beam (λ = 1.55 Å), on a HUBER precession camera equipped with a He path. Exposure time = 3 min, crystal-to-film distance = 200 mm. (Left) 1° rotation photograph of 0kl zone, 680 × 920 Å; (Right) 1° rotation photograph of h0l zone, 360 × 920 Å.
fraction patterns from all of the zones (Fig. 5) without manipulating the crystals.

Oriented arcs and distinct spots, with spacings similar to those measured from diffuse diffraction patterns of ribosome gels and extracted rRNA, and extending to 3.5 Å, have been detected on several diffraction patterns of single crystals as well as on those of samples containing large numbers of microcrystals. For aligned crystals the average arc length is ±30°. Such patterns may arise from partial orientation of the nucleic acid component within the particle.

Recently we have developed procedures for growing crystals from this source (i.e., 50S subunits from *B. stearothermophilus*) using nonvolatile materials. Currently we can grow well-shaped crystals under conditions similar to the physiological ones. These crystals grew from very low concentrations of polyethylene glycol, in the presence of the ions which are essential for their activity and integrity. The crystals, shaped as wide needles (0.3 × 0.1 × 0.05 mm) are packed rather densely in cells of \( a = 280 \, \text{Å}, \, b = 385 \, \text{Å}, \) and \( c = 526 \, \text{Å}, \) diffract to better than 11 Å, and show moderate degree of mosaic spread (1–1.5°). Crystallographic data were collected at cryogenic temperature from crystals soaked in viscous solutions of polyethelene glycol containing an antifreeze (ethylene glycol). These conditions were suitable for native crystals, for crystals of the mutated ribosomes (missing protein BL11, see below), and for a complex containing the 50S subunits as well as lys-tRNA + polyLys (18–20mers).

Crystals of the 50S subunits from *H. marismortui* grow at 19° by vapor diffusion and seeding as thin plates with a maximum size of 0.6 × 0.6 × 0.2 mm (Fig. 4). Although fragile, they can be manipulated. Among the crystals of the ribosomal particles, they are the most ordered and rigid. They diffract to a resolution of better than 6 Å (Fig. 6). They have relatively small, compactly packed unit cells (of 214 × 306 × 594 Å, Table (c) Electron micrographs of positively stained (2% uranyl acetate) thin sections of crystals similar to those shown in (a) that have been fixed in 0.2% glyceraldehyde and embedded in resin ERL 4206. Optical diffraction patterns are inserted. (Right) Micrograph showing the characteristic “open” packing of this crystal form. The orthogonal choice of axes corresponds to the 680 × 920 Å zone observed in the X-ray diffraction patterns. Lattice spacing calculated from optical diffraction: 670 × 850 Å. (Left) Section approximately perpendicular to that shown on the right. Repeat distances measured from optical diffraction: 330 × 1050 Å. This corresponds to the hOl zone (360 × 920 Å) in the X-ray patterns.

Fig. 6. (a) A crystal of the 50S ribosomal subunits of \textit{H. marismortui} obtained under similar conditions of Fig. 4(d). (b,c) 1° rotation patterns of a crystal shown in (a) aligned along the major crystallographic axes. The patterns were obtained at 4° with synchrotron radiation (X31 station at EMBL/DESY operating at 5 GeV, initial current 60–80 mA). The X-ray beam of wavelength 1.48 Å was collimated by vertical and horizontal slits (0.3 × 0.3 mm). Exposure time = 45 min, crystal to film distance = 150 mm. (b) 0kl zone; (c) hko zone (obtained at a spindle setting at 90° away from the left pattern).

I), in contrast to the open structure of the large crystals of \textit{B. stearothermophilus} (Table I and Fig. 5).

Crystallographic data have been collected from crystals of these subunits, from their complex with tRNA+polyPhe(18–20mers), and from crystals soaked in solutions of 11-gold or tetrairidium clusters (see below). In all cases crystals were immersed in an inert hydrocarbon, mounted on tiny glass spatulas, and flash-cooled in a stream of liquid nitrogen at 85–90 K.

\textit{The Phase Problem}

To represent a three-dimensional structure by Fourier synthesis it is necessary to sum all the waves (reflections) present in the diffraction pattern of the crystal. Each reflection is characterized by its direction,
intensity, and phase. What keeps it from being a trivial computational problem is that phases cannot be directly observed. The most common method in protein crystallography to derive phases is multiple isomorphous replacement (MIR). For application of this method, a set of at least two heavy-atom derivatives has to be prepared. These are crystals isomorphous to the native ones but including an additional electron-dense compound which must sit in one or a few specific sites at the same locations in each unit cell. The compounds commonly used for heavy-atom derivatization in conventional protein crystallography contain heavy metals, such as platinum, mercury, and gold. Heavy-atom derivatives are obtained by soaking the native crystals in a solution containing such a compound, or by binding of a heavy-atom ligand to a specific group on the protein prior to its crystallization.

For an object as large, asymmetric, and complex as the 50S ribosomal subunit, it is necessary to use extremely dense and compact compounds. Heavy-atom clusters are most suitable for this purpose. However, due to its size and complexity, the surface of the ribosomal subunit is a composite of a variety of potential interaction sites for such clusters. Therefore, on soaking in solutions of a heavy-atom cluster, the latter may be attached to multiple sites on the ribosomes and complicate phase determination or make it impossible. Thus, in order to obtain usable heavy-atom derivatives, these clusters should be covalently bound to a few specific sites on the ribosomal particles. This may be achieved by direct interaction of a heavy-atom cluster with chemically active groups such as SH or the ends of rRNA, on the intact particles prior to crystallization, or by covalent attachment of a cluster to tailor-made carriers which bind to one or a few specific sites on ribosomes.

An example of a suitable candidate for this purpose is a gold cluster, $\text{Au}_{11}(\text{CN})_3[P(\text{C}_6\text{H}_4\text{CH}_2\text{NH}_2)_3]_7$, in which the gold core has a diameter of 8.5 Å. This compound has been prepared as a monofunctional labeling reagent. Appropriate carriers may be antibiotics, DNA oligomers complementary to exposed single-stranded rRNA regions, and Fab molecules specific to ribosomal proteins. Since most of the interactions of these materials are characterized biochemically, the crystallographic location of

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68 W. Jahn and S. Weinstein, unpublished observations.
the heavy-atom compounds will not only be used for phase determination but will also reveal the location of specific sites on the ribosomes. Alternatively, these clusters may be attached to chosen sites on isolated ribosomal components which will subsequently be incorporated into particles in which they are missing.

A mutant of *B. stearothermophilus* which lacks protein L11 was obtained by growing cells in the presence of thiostrepton at 60° (see above). The 50S mutated ribosomal subunits crystallize in two and three dimensions under the same conditions as, and are isomorphous to, those obtained from the 50S ribosomal subunits of the wild type. This shows that L11, the missing protein, is not involved in crystal forces in the native crystals. Furthermore, binding of N-ethylmaleimide to the SH group of protein L11 does not reduce the activity and crystallizability of the reconstituted modified particles.

Phase information may also be obtained by neutron diffraction, as performed for the nucleosomes, and by direct methods. As mentioned above, results of electron microscopy are invaluable for phase determination. The model of the 50S particle, obtained by three-dimensional image reconstruction, could be placed in the crystallographic unit cell using crystal-packing information derived from the electron micrographs of thin sections of the same crystals (Fig. 5), as performed for nucleosomes and viruses. In addition, the application of real- and reciprocal-space rotation searches should be feasible by taking advantage of the noncrystallographic symmetry within the asymmetric units.

A model obtained at medium resolution could be used, together with high-resolution information obtained from crystallographic studies of isolated individual ribosomal components, for iterative phase determination by molecular replacement methods, assuming that the conformations of crystallized isolated components are sufficiently similar to their conformations within the particle.

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72 M. Kimura, unpublished observations.
73 S. Weinstein, unpublished observations.
Concluding Remarks

The methods of crystallography and high-resolution three-dimensional image reconstruction have been introduced recently to the field of ribosomes. However, all elements that should assure the success of these techniques have already been demonstrated. Furthermore, a vast amount of knowledge, concerning the chemical, biological, physical, and genetic properties of ribosomes, from which structure analysis should benefit enormously, has been accumulated. It is expected that the results of the structural studies will lead to a better understanding of the role of ribosomes in protein biosynthesis.

[7] Neutron-Scattering Topography of Proteins of the Small Ribosomal Subunit

By Malcolm S. Capel and V. Ramakrishnan

The quaternary organization of the proteins of the *Escherichia coli* small ribosomal subunit has been investigated by nearly every possible experimental approach, including immunoelectron microscopy,1 bifunctional chemical cross-linking,2 fluorescence energy transfer,3 and neutron scattering.4-6 The results of all of these different mapping efforts are surprisingly consistent with one another, and provide a nearly complete understanding of the quaternary organization of the proteins of the 30S ribosomal subunit.7,8 In this chapter we describe the low-angle neutron-scattering methods we have employed to determine the three-dimensional configuration of the proteins of the 30S ribosomal subunit of *E. coli*. The basic strategy for data collection and analysis has been discussed before in

8 P. B. Moore, M. S. Capel, M. Kjeldgaard, and D. M. Engelma, in "Structure, Function and Genetics of Ribosomes" (B. Hardesty, ed.). Springer-Verlag, New York, in press.