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CRYSTALLIZATION, ELECTRON MICROSCOPY AND THREE-DIMENSIONAL RECONSTRUCTION STUDIES OF RIBOSOMAL SUBUNITS

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Ribosomes are the site of protein biosynthesis, hence their chemical, physical and biological properties have been extensively studied. These studies have shed light on the process of protein biosynthesis, although, due to the lack of a reliable structural model for the ribosome, a full understanding of the molecular mechanism is still not possible.

Diffraction methods, such as X-ray crystallography or three-dimensional image reconstruction from electron micrographs offer direct structural information, hence these are the most powerful techniques for obtaining reliable molecular models. Usage of these techniques depends on the availability of crystalline materials, and we have directed our efforts to obtain them.

Two-dimensional periodic organization of eukaryotic ribosomes occurs under special conditions *in vivo* (Kühlbrandt and Unwin, 1980, Barbieri, 1979, O'Brien et al., 1980, Kress et al., 1971). Small ordered sheets of ribosomal subunits from bacterial sources have also been obtained *in vitro*, (Lake, 1980). Recently we were able to extend the degree of organization and obtained well-ordered three-dimensional crystals of the large subunit of *B. stearothermophilus in vitro*. The crystals were grown employing a slightly modified version of the vapour diffusion technique in hanging drops and in capillaries (Yonath et al. 1980, 1982a). The crystallization drops which contained the ribosomal subunits (Cronenberger and Erdmann, 1975) and Mes or Hepes buffers (pH 6.3-7.5), were equilibrated with organic solvents in the presence of 0.5-0.9 M NaCl and 6mM β-mercaptoethanol in the reservoir (at 3-7°C).

The crystalline ribosomal particles are intact and functionally active. Slight variations in the crystallization conditions and in the preparation of the subunits resulted in the appearance of several packing modes. The various ordered forms are described by Yonath et al., (1982b), those used for electron microscopical studies are given in Table 1 and shown in Figs. 1-3. It should be mentioned that variations of up to 30% in the apparent cell constants may result from shrinkage during preparation for electron microscopy. Thus, crystals #1, and #2 belong, probably, to the same crystal form.

The prospects for three-dimensional structure determination seemed promising, since we were able to detect, so far for one crystal form (#1), X-ray diffraction patterns to 0.35nm for "powder" and 1.0nm for "still"

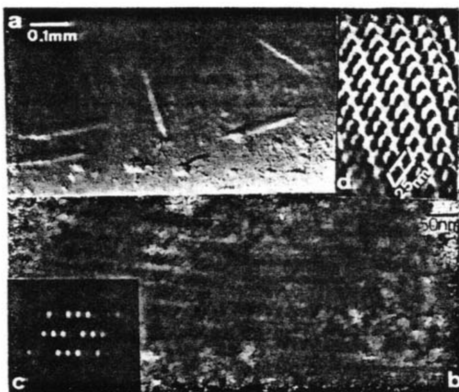

Fig. 1 (a) Crystals of form #1 as seen by a light microscope. (b) Electron micrograph of a thin section of (a); its (c) Optical diffraction pattern and (d) Computed filtered image (the unit cell is depicted).

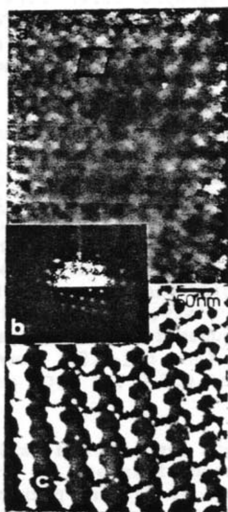

Fig. 2 (a) Electron micrograph of a thin section of a crystal form #3; its (b) Cptical diffraction pattern and (c) Computed filtered image (The unit cell is depicted).

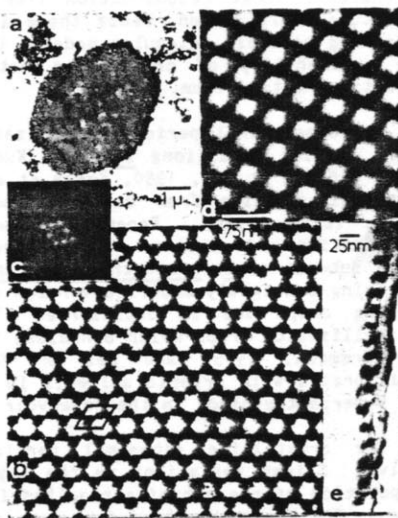

Fig. 3(a) Electron micrograph of a thin section of a whole crystal of form #4. (b) An enlarged view of this section, its (c) Optical diffraction pattern and (d) Computed filtered image (The unit cell is depicted). (e) Sideways on section of the grid shown in (b).

pictures of cross-linked single crystals with detectable spacings of 15.4nm and 26.1nm. At present, the crystals are too small for efficient crystallographic data collection, but are large enough to be studied by electron microscopy.

The crystals are too thick to image directly in the microscope but thin Epon-embedded sections of these crystals can be treated as two-dimensional ordered sheets. Unit cell constants and symmetry in projection can be obtained from micrographs of such sections. Three-dimensional image reconstruction of tilted specimens can also yield useful information about crystal packing as well as three-dimensional representation of the stain distribution for the ribosomal particles. Such studies are of interest not only because they lead to a low resolution model of the stained portion of the ribosomal subunits, but also, in view of the size and the complexity of these particles, they are of value as an intermediate step toward structure determination at higher resolution.

Table 1

Crystals used for reconstruction studies

Crystal form	Projected cell dimensions (nm)	Quality for image reconstruction
#1	130 x 254; $\gamma = 95°$	good
#2	156 x 288; $\gamma = 97°$	fair
#3	260 x 288; $\gamma = 105°$	fair
#4	450 x 450; $\gamma = 120°$	very good

Crystals were fixed in 0.2% glutaraldehyde and embedded in resin ERL 4206. Thin sections (~50nm) were positively stained with 2% uranyl acetate. Electron microscopy was carried out in a Jeol 100B at 10,000, 30,000, and 60,000 magnifications.

Crystalline areas were selected in the electron microscope by searching for regions where the appearance of the lattice was constant over an area of more than a 100 unit cells. For ribosomes, the section thickness is close to one asymmetric unit, so that a section cut at an angle to the unit cell edge will result in a long range modulation. Such areas were avoided. Selected areas of electron-micrographs which showed the best resolution were digitized on an Optronics (P-1000) densitometer, as 512x512 points on a square raster of 50µm. Fourier transforms of the digitised images were computed on 512x512 matrix. The calculated diffraction patterns were indexed by hand and the reciprocal lattice located by least sqares fitting to the strongest diffraction maxima. Structure factors were then extracted from the stored Fourier map as the peak amplitudes and phases values. These were used to calculate two-dimensional noise-filterd and avaraged images, or stored for combination of filtered images (see below).

Normally, in carrying out a three-dimensional reconstruction of a thin crystal, tilted views of several crystals at different orientations to the tilt axis are combined (Henderson and Unwin, 1975). This reduces the avarage electron exposure and anables data to be collected over all directions of the reciprocal space. The maximum tilt angle is $+60^{o}$, so that a cone of data will always be missing around the direction perpendicular to the plane of the crystal. With thin sections, it is not possible to combine views of different crystals since the thickness and the exact sectioning direction relative to the unit cell is variable. However, embeded sections are more resistant to elctron damage, thus it is possible to collect data over a full range of the reciprocal space (excluding the missing cone) by tilting a single crystal around several different directions. Micrographs were then taken over a tilt range of $\pm 50^{o}$, with intervals of $5-10^{o}$, around four (or two) axes, 45° (or 90°, respectively) apart, in the plane of the section. Each set of data, consisting of between 34 to 59 images at different tilts and at different angles to the tilt axis, was then refined against all previously accumulated phases to a common phase origin in reciprocal space. Data sets for which the rms phase error was greater than 40° were rejected. Continuous variations of both amplitudes and phases were plotted as a function of the vertical (Z^*) direction of the section, to allow sampling in l at intervals of 1/50 nm (Fig. 4). The resulting data (in h,k, and l) were Fourier transformed to give a three-dimensional density distribution of the stained portion of the particle. Details of data collection and processing are given in Table 2.

Table 2

Details of Data Collection

Crystal form	No. of tilt axes	total no. of tilts taken	no. of tilts used	no. of reflections use for Fourier transform	Typical residual of phase error	Maximum resolution (nm)
#1	2	34	24	165	17	5.0
#2	2	34	24	59	20	6.0
#3	2	38	26	58	20	7.0
#4	4	59	52	353	22	7.5

Crystals of form #1 are large enough for preliminary X-ray diffraction studies (Fig. 1a), but disintegrate considerably upon preperation for electron microscopical studies (Fig. 1b). Therefore, the available areas for digitizing are not of optimal size. However, as seen in Fig. 1c and Table 2, their optical diffraction patterns extend to about 5nm, which is the best resolution of any of the crystal forms. Three-dimensinal reconstruction from this crystal form gave a model (Fig. 5a), consistent

in size with that expected for the large ribosomal subunit, but somewhat different in shape from that seen in negatively stained images. It shows clearly two domains of high density which may result from positive stain preferentially contrasting selected parts of the structure.

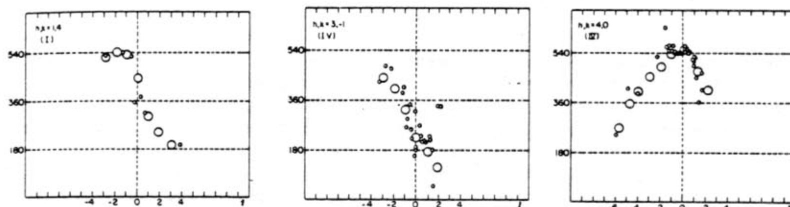

Fig. 4. Representative values of phases (o) determined along reciprocal lattice lines from micrographs tilted to the incident beam by angles up to ±50. The large circles are those obtained by sampling in 1 at reciprocal lattice intervals of 1/50 nm.

The available ordered areas of the crystals of forms #2 and #3 (Fig. 2) are rather small (~75 unit cells each), but image-reconstruction studies led to a two-domain structure, similar to that of form #1. A superposition of the outlines of the stain distribution in crystals forms #1, #2 and #3 is shown in Fig. 5b.

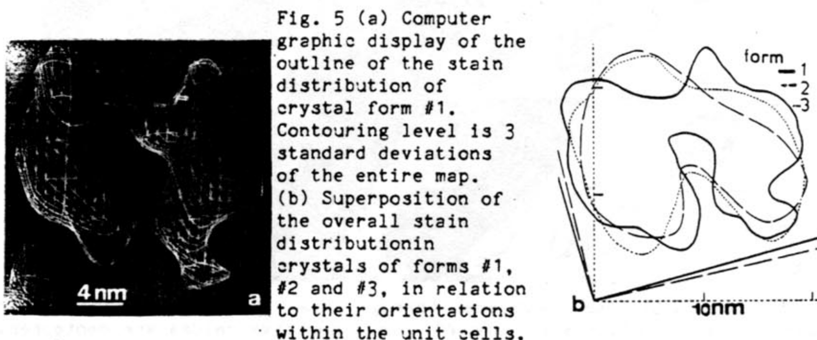

Fig. 5 (a) Computer graphic display of the outline of the stain distribution of crystal form #1. Contouring level is 3 standard deviations of the entire map. (b) Superposition of the overall stain distributionin crystals of forms #1, #2 and #3, in relation to their orientations within the unit cells.

The crystals of form #4 are still fairly small (0.03mm diameter), but they survive extremely well during the preparation for electron microscopy so that the entire crystal could be sectioned (Fig. 3a). It was also possible to cut the section used for image reconstruction in a direction perpendicular to the plane of the section (Fig. 3e). This gives a direct measure of the section thickness and the distribution of the stained material perpendicular to the normal view, which is in good agreement with the thickness calculated from the reconstruction studies (see below). Although these crystals appear to be hexagonally packed, the diffraction patterns (Fig. 3c) show that the 6-fold symmetry does not hold even for the second order. Phase data indicates that lower symmetry, such as P2,

breaks down for higher order reflections. This could result from sectioning direction that was not absolutly perpendicular to the symmetry axis. Since there was no evidence either in the computed diffraction patterns or in the filtered images of the tilted views for a true crystallographic 6- (or 3-) fold symmetry axis, we treated it as being triclinic.

Sections through the three-dimensional map and the projected view of the reconstructed stain density in crystal form #4 are shown in Fig. 6. Regions of high as well as of low stain density are easily seen. Since the sections are positively stained one should detect the distribution of the stain within the ribosomal particles rather than their surface as is the case with negatively stained material. This could reveal the location of the chemical entities that interact with the positively charged stain (uranyl acetate), such as the rRNA chains, and to lesser extent, the ribosomal proteins, which are mainly positively charged. Regions that appear as "empty" or that show a low stain density may either be less exposed to the stain, or composed of the solvent or of a material, that due to its chemical nature, does not interact with it. To clarify this point, we are studying sectioned crystals of this form, using different positive stains.

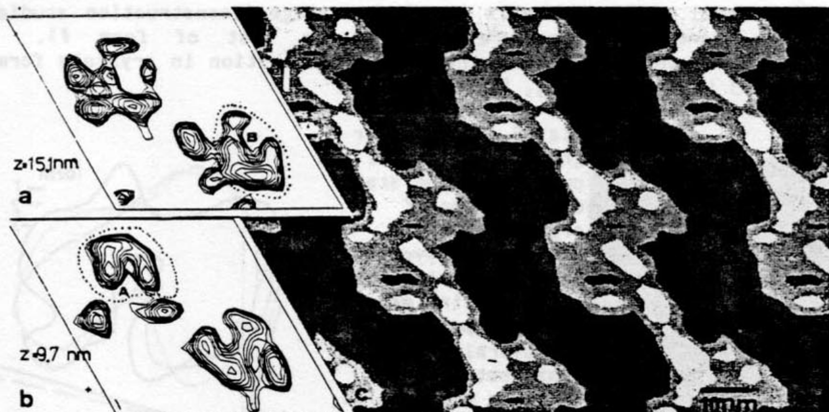

Fig. 6 (a,b) Two sections through the three-dimensional stain distribution map of crystal form #4. Only positive values are contoured. The lowest level correspond to 2.5 standard deviations of the map, and the increment between contours correspond to 1.2σ. A and B are the outlines of two particles as seen on these sections. (c) Projected view of the reconstructed stain density.

Fig. 7 shows the three-dimensional model of the entire unit cell of crystal form #4. The volume of the reconstructed density fits well with that of four large ribosomal subunits, and the unit cell seems to be built of two parts, each consisting of two subunits, related by a pseudo two-fold rotation axis.

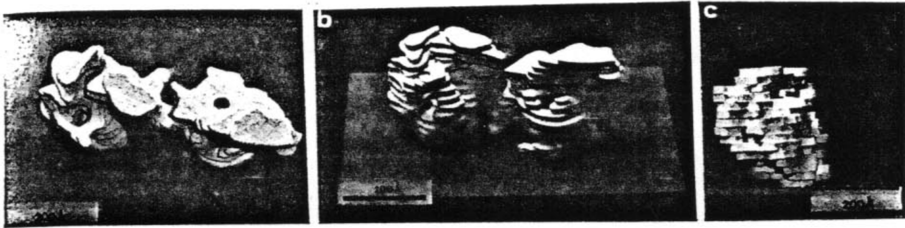

Fig. 7 Three-dimensional model of the stain distribution within the unit cell of crystal form #4. (a) Top view. (b,c) Side views.

In general, as a result of the missing cone, models obtained by three-dimensional image reconstruction studies from tilted two-dimensional arrays are not well determined along the direction perpendicular to the plane of the section. Since in this case, the ribosomal particles are located in several orientations in the different crystal forms, we hope that avaraging of the obtained structures, will result in an isotropically reliable model for the stain distribution. Work along these lines is in progress.

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