1.7 Cryocrystallography of native and derivatized ribosomal crystals

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Ribosomes are the universal supramolecular assemblies responsible for the translation of genetic information, encoded in mRNA, into proteins. A typical bacterial ribosome contains more than 250 000 atoms, has a molecular weight of about 2.3×10^6 and a sedimentation coefficient of 70S. It is composed of two subunits of unequal size (small: 30S, molecular weight 0.85×10^6, and large: 50S, molecular weight 1.45×10^6), which associate upon the initiation of the biosynthetic process. It contains three chains of rRNA (about 5500 nucleotides), accounting for two-thirds of its mass and 57–73 different proteins, depending on the source of the ribosome.

Of all the intracellular organelles, only ribosomes have thus far been crystallized. X-ray crystallography has recently seen major advances in the sophistication, effectiveness, efficiency, and accuracy of data collection and interpretation. Nevertheless, being ribonucleoprotein assemblies, which are notoriously flexible, unstable, and routinely prepared as conformationally mixed populations, even the best crystals of ribosomes dictate unconventional conceptual and technical approaches.

Systematic exploration of crystallization conditions combined with sophisticated seeding has led to reproducible growth of ordered three-dimensional crystals of ribosomal particles from halophilic and thermophilic bacteria, diffracting best to 2.9 Å resolution (Fig. 1). In addition, complexes of ribosomes with components of protein biosynthesis, mimicking defined functional states, as well as mutated and chemically modified ribosomal subunits have been crystallized (Table 1). In all cases, the crystalline ribosomal particles retain their integrity and biological activity for long periods despite their natural tendency to disintegrate rapidly.

In this chapter we will describe our progress in collecting and processing crystallographic data from ribosomal crystals, highlight our special problems, and discuss our strategy.
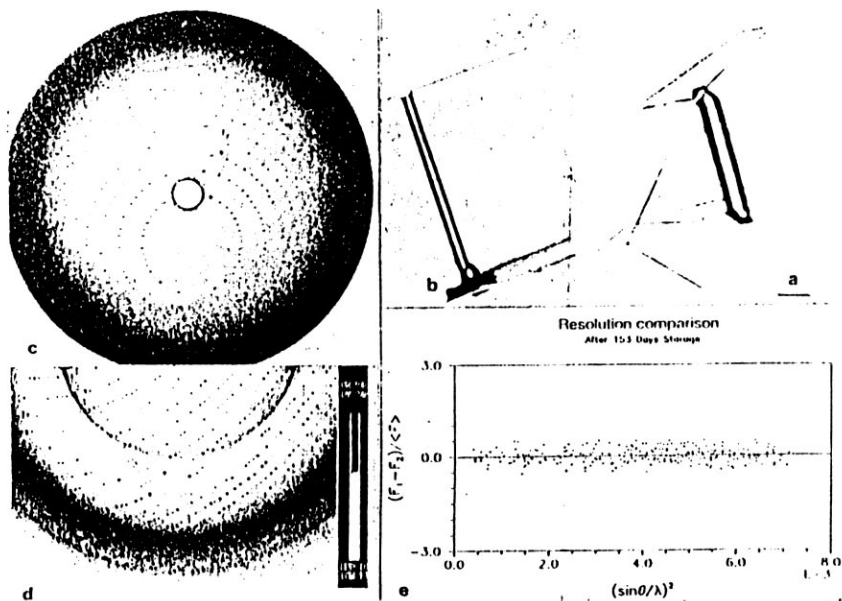
Fig. 1 (a) Crystals of the large ribosomal subunits from *Haloarcula marismortui*, diffracting to 2.9 Å resolution, grown at 19°C, by vapour diffusion coupled with seeding, from 6–8 µl of: 5 mg/ml 50S subunits, 1.2 M potassium chloride, 0.5 M ammonium chloride, 0.005 M magnesium chloride, 0.001 M cadmium chloride, and 5–6 per cent polyethyleneglycol (6000), at pH=5.6 equilibrated with a 1 ml reservoir of 1.7 M KCl and all the other components of the drop. Scale bar, 0.1 mm. (b) A typical double-layer ('sandwich') perpendicular spatula, used for mounting shock-frozen crystals, similar to those shown in (a). (c) 1° rotation photograph of a crystal similar to that shown in (a) kept in 3 M potassium chloride, 0.5 M ammonium chloride, 0.005 M magnesium chloride, 0.001 M cadmium chloride, and 8 per cent polyethyleneglycol (6000), at pH=5.6. Before cooling the crystal was soaked for 15 min in a solution containing the above storage components and 18 per cent ethyleneglycol. The pattern was obtained on film, by exposing for 8 min at 90 K at Station F1/CHESS, operating at about 5.3 Gev and 50–80 mA. The crystal to film distance was 220 mm; wavelength = 0.9091 Å. (d) 1.5° Weissenberg image of a crystal similar to that shown in (a). The pattern was obtained in 24 min at 90 K at Station BL6/PF/KEK, operating at about 2.5 Gev and 230 mA. The crystal to film distance was 573 mm; wavelength = 1.00 Å. (e) Comparisons of data collected from a fresh crystal (1) of 50S ribosomal subunits of *B. stearothermophilus*, and after 2 days of irradiation and 153 days of storage (2).

Table 1 Ribosomal crystal

Source*	Sedimentation coefficient†	Grown from‡	Cell dimensions (Å)	Space group	Resolution (Å)§
T.t.	70S	MPD	524 × 524 × 306	$P4_12_12$	~20
T.t. + mRNA and tRNA¶	70S	MPD	524 × 524 × 306	$P4_12_12$	~15
T.t.	30S	MPD	407 × 407 × 170	$P42_12$	7.3
H.m.	50S	PEG	210 × 302.5 × 571	$C222_1$	2.9
T.t.	50S	AS	495 × 495 × 196	$P4_12_12$	8.7
B.st.‖	50S	A	360 × 680 × 920	$P2_12_12$	~18
B.st.‖,**	50S	PEG	300 × 547 × 38‡	114°, C2	~11

* T.t., Thermus thermophilus; H.m., Haloarcula marismortui; B.st., Bacillus stearothermophilus.

† 30S. Small ribosomal subunits: 50S, large ribosomal subunits: 70S, whole ribosomes.

‡ Crystals were grown by vapour diffusion in hanging drops from solutions containing methyl-pentane-diol (MPD), polyethylene-glycol (PEG), ammonium sulfate (AS), or low-molecular-weight alcohols (A). The exact conditions are given in Berkovitch-Yellin et al. (1992).

§ The resolution given here is the 'useful resolution', the limit at which a significant amount (above 40%) of data could be properly evaluated. The 'potential resolution' is the highest Bragg spacing at which sharp diffraction spots are consistently observed.

¶ A complex including 70S ribosomes, two molecules phetRNA:phe, and an oligomer of 35 uridines (as mRNA).

‖ Same form and parameters for crystals of large ribosomal subunits of a mutant (lacking protein BL11) of the same source and for modified particles with an undecagold cluster.

** Same form and parameters for crystals of a complex of 50S subunits, one tRNA molecule, and a segment (18-20 mer) of a nascent polypeptide chain.

Description and discussion

Introducing cryocrystallography

Due to the large unit cell dimensions and the extremely weak diffracting power of the ribosomal crystals, all the X-ray crystallographic analysis must be performed with intense synchrotron radiation. At ambient temperatures, the radiation damage suffered by these crystals is so severe that all reflections beyond Bragg spacings of 15–18 Å decay almost instantaneously. This extreme sensitivity led initially to the incorrect conclusion that the measurable diffraction of all the ribosomal crystals was limited to that resolution (Yonath *et al.* 1984). Thus, for collecting the higher-resolution terms, only one exposure could be obtained from each individual crystal and precise alignment was impossible. The combination of randomly oriented crystals, a high mosaic spread, and single exposures resulted in repeated failures to collect complete data sets, even when hundreds of crystals were exposed.

The radiation damage of the ribosomal crystals was virtually eliminated by collecting the crystallographic data at cryogenic temperatures (about 90–100 K) from shock-frozen crystals. An experimental procedure was designed to accommodate the unique features of the ribosomal crystals: anisotropic morphology (at least one very thin dimension); extreme softness; and high fragility. Prior to cooling, the crystals are transferred to media similar to their original stabilizing solutions, but with a somewhat higher viscosity. Often a cryosolvent has to be added to avoid the formation of ice crystals. A thorough empirical search to establish individual pre-cooling treatment for each crystal type is essential, the variables being the type and compositions of the added materials, the fashion of addition, and the time course of the treatment.

To protect the crystals from drying and from bending stresses resulting from surface tension effects, a variety of microspatulas have been constructed. They consist of a thin glass rod to which single or double layers ('sandwiches') of thin glass plates are glued in selected directions, allowing mounting at desired orientations (Fig. 1; Hope *et al.* 1989). A crystal, immersed in a very small amount of solvent, is placed on top of the single spatula or between the layers of a 'sandwich', and immediately plunged into liquid propane at a temperature near its melting point, to enable vitrification of the solvent surrounding the crystal. A special device was constructed for rapid transport of the crystal to the X-ray camera, where it is transferred from the liquid propane into the nitrogen stream at around 100 K, and kept there throughout data collection. Under these conditions irradiated frozen ribosomal crystals show no signs of decay over periods longer than the time needed to collect a complete diffraction data set (usually a few days). To resume interrupted diffraction experiments, we constructed a device for preserving irradiated crystals in solid propane/liquid nitrogen chambers for extremely long periods, even a few years.

A quantitative assessment of the effect of shock-freezing on the resolution

and the mosaic spread is still impossible, mainly because the same crystal cannot be exposed both at ambient and at cryotemperatures, due to the irreversibility of the effects of radiation damage. However, for ribosomal crystals, it is clear that the mosaicity of properly shock-cooled crystals is conserved throughout the shock-freezing procedure. The resolution limits of the diffraction patterns measured at cryogenic temperatures should not be higher than those obtained at ambient temperature, since these limits reflect the intrinsic conformational heterogeneity of the crystal. On the contrary, it is likely that the various steps in the cooling procedure, with their associated thermal, osmotic, and mechanical stresses, may lead to a deterioration of crystal order. However, as the frozen crystals can be irradiated for a long time, an apparent improvement in resolution was observed, resulting from the detection of a larger number of higher-resolution reflections, which are usually very weak. To estimate the influence of the storage at cryotemperature, the diffraction data from a fresh crystal were compared with data collected after 24 h of irradiation and after 153 days of storage in solid propane. In both cases no intensity changes were detectable (Fig. 1e).

Specific concerns in data collection and evaluation

Data were collected mainly using the screenless rotation method, and occasionally using the Weissenberg technique. The choice of the rotation range per exposure is usually a compromise between the unit cell dimensions, the resolution limit, the diffracting power, and the mosaicity. For ribosomal crystals it is largely influenced by the latter, which is of a significant magnitude even for the best crystals. The weak diffraction power of the ribosomal crystals necessitates very long exposures (e.g. 8–24 min/degree at BL6/PF/ KEK or 8–10 min/degree at F1/CHESS) leading to exceptionally high background and limiting the rotation range. Consequently, the advantage of the option of translation on spot separation of the Weissenberg camera was minimized. This is rather unfortunate, since even under these severe limitations, the Weissenberg data are of a quality comparable to those obtained by the rotation method (Table 2).

A common characteristic in the diffraction patterns of ribosomal crystals is the extremely steep descent of intensities as a function of resolution. Originally we assumed that imaging plates or five-film packs, could cover the entire range of intensities. However, exposures sufficient for obtaining measurable intensity for the weakest reflections resulted in oversaturated reflections, even on imaging plates or the fifth film in a pack. Therefore, typically, several data sets are being collected from each crystal, with rotation ranges and exposure times optimized for different resolution shells. This is feasible, since at cryotemperature the ribosomal crystals can be exposed for virtually unlimited periods. A special emphasis is given to the accurate measurement of the low-resolution reflections, since these have been found to be of

Table 2 Results of evaluation (to 6.4 Å) of three sets of crystallographic data from crystals of 50S ribosomal subunits of *Haloarcula marismortui*, diffracting to 2.9 Å, with unit cell dimensions of 212 × 302.5 × 571 Å

	Data set		
	(a)*	(b)†	(c)‡
Wavelength (Å)	0.9191	0.9191	1.0
Rotation range/exposure (°)	10	1.0–1.5	2 (0.5 overlap)
Exposure time (per rotation)	100 s	8–12 min	24 min
Crystal-to-film distance (mm)	573	220	573
Sigma cut-off	2.5	2.5	2
Mosaic spread (°)	0.2	0.2–0.3	0.4
Evaluated reflections (no.)	2367	253 673	83 994
Fully recorded reflections (no.)	2061	57 829	83 994
Partially recorded reflections (no.)	306	195 601	
Unique reflections (no.)	389	27 561	30 719
$R_{merge}(I)$ (%)	4.71	9.53	10.56

Data set (a)		Data set (b)		Data set (c)	
Resolution shell (Å)	Completeness	Resolution shell (Å)	Completeness	Resolution shell (Å)	Completeness
316.0–58.1	0.88	316.0–12.9	0.91	316.0–12.9	0.92
59.1–46.1	0.82	12.9–10.3	0.88	12.9–10.3	0.89
46.1–40.3	0.87	10.3–8.9	0.83	10.3–8.9	0.85
40.3–36.6	0.68	8.9–8.1	0.78	8.9–8.1	0.84
36.6–34.0	0.71	8.1–7.6	0.72	8.1–7.6	0.75
34.0–32.0	0.56	7.6–7.1	0.69	7.6–7.1	0.72
32.0–30.4	0.56	7.1–6.7	0.63	7.1–6.7	0.70
30.4–28.1	0.58	6.7–6.4	0.52	6.7–6.4	0.69

*Set (a) data were collected to 28 Å with synchrotron radiation at cryotemperature using the rotation method on film at FICHESS, Cornel! University.
†Set (b) data were collected to 3.5 Å with synchrotron radiation at cryotemperature using the rotation method on film at FICHESS, Cor nell University.
‡Set (c) data were collected to 3.0 Å with synchrotron radiation at cryotemperature using the Weissenberg method on imaging plate a Beamline BL6. Photon Factory. Japan.

significant value in tracing molecular envelopes in low-resolution electron-density maps, the first step of low-resolution phasing.

So far, the unit cell dimensions of the ribosomal crystals could be determined only by synchrotron radiation at cryotemperatures. In some instances, striking isomorphism was observed. In others, a significant non-isomorphism is evident even between native crystals from the same batch. Currently, it is not clear whether the variability in unit cell dimensions is an inherent property or induced by the cooling. In addition, crystals that appear isomorphous in their cell dimensions may tolerate some internal variability in packing. No matter what is the cause for the apparent (or real) lack of isomorphism, it may complicate phasing by multiple isomorphous replacement (MIR) or other methods based on differences in the magnitudes of structure factors. Therefore we seek phasing procedures that rely on data collected from one crystal. These may be computational (e.g. maximum entropy) and/or experimental (e.g. multiwavelength anomalous dispersion (MAD), using a derivatized crystal successively irradiated at different wavelengths).

The high-resolution diffraction data sets contain several hundred thousand reflections. For example, in the shell 6–49 Å, more than 250 000 reflections were measured, yielding 27 560 unique ones and in the shell 3.5–10.0 Å, there are 128 500 measurements, with 102 970 unique reflections. It was found that evaluation of the ribosomal data is still not a routine task, although some special computational tools have been developed specifically for this aim. To maximize the quality of the data and to avoid misindexing of the populated patterns, an extremely high degree of precision of all geometrical parameters is crucial. This is achieved by processing the data using an interactive computer graphic display, introduced by us specifically for this purpose. As a result most of the data so evaluated are of a reasonable degree of completeness and of quality comparable to that obtained from crystalline proteins of average size; the typical values for R-merge (I) are in the range 4.5–9.8 per cent (see also Table 2).

Specific labelling by an undecagold cluster

Due to the large size of the ribosome, ideal compounds for its derivatization should consist of a compact cluster of a large number of heavy atoms, linked directly to each other. Since ribosomal particles have an extremely large and complex surface area, we designed procedures for quantitative derivatization at specific sites before crystallization. This approach requires sophisticated synthetic techniques and time-consuming purification procedures, but offers a higher chance of obtaining single-site derivatives.

A water-soluble monofunctional undecagold cluster (molecular weight, 6200), specific for covalent binding to free sulfydryls, was prepared (Weinstein et al. 1989), and its potential phasing power for MIR and MAD phasing was demonstrated by simulation studies. To overcome limitations in accessibility imposed by the bulkiness of the gold cluster, the chemically active

moiety was attached to the cluster via a handle of substantial length (18–20 Å). The flexibility of this arm facilitates almost quantitative binding to the ribosome, but, at the same time, may lead to a loss in phasing power.

To increase the accuracy of phasing at higher resolution, a rather short (about 4 Å) and rigid arm with a structure similar to the peptide bond was prepared. The improved cluster was used for quantitative binding to natural or engineered sulfydryl groups of isolated ribosomal proteins, which are less bulky than the whole ribosome. The modified proteins were, in turn, incorporated in ribosomal core particles lacking them (Weinstein et al. 1989). The fully derivatized ribosomal particles were crystallized, under similar conditions to those used for crystallizing the particles modified with the long-arm-cluster, which yielded crystallographic data of reasonable quality to 14 Å resolution.

The cluster was also bound to tRNA^{phe} from *Escherichia coli*. Using radio-activity it was established that the gold-cluster derivatized tRNA molecules bind to 70S and 30S particles and can be aminoacylated by their cognate synthetase with the same yield as the native molecules. As tRNA is part of several crystallized complexes of ribosomal particles (Table 1; Hansen et al. 1990; Berkovitch-Yellin et al. 1992), its gold-cluster derivative may be used for phasing the diffraction data from complexes containing them.

In attempts to modify the halophilic subunits, four ribosomal proteins were selectively detached by dioxane. The cores lacking three of these proteins were crystallized. One of the removed proteins, HL11, binds -SH reagents, but in contrast to the native one, the modified protein could not be incorporated into the core particles. In this way 50S subunits depleted of protein HL11 were formed. These crystallize under the same conditions as native subunits and diffract to 10 Å resolution. Data were collected to this limit.

Concluding remarks

We demonstrated here that crystallographic studies on intact ribosomal particles are feasible, despite the fact that the application of conventional macromolecular crystallographic techniques is not straightforward and that considerable effort must be devoted to introducing new approaches and alternative experimental procedures. Of special significance are: the development of innovative procedures for crystallization and for seeding; the introduction of cryotemperature techniques for eliminating extensive radiation damage; the introduction of interactive graphics for evaluation of the crystallographic data; and the combination of metallo-organic biochemistry, genetic manipulations, and functional studies to enable specific labelling of the ribosomal crystals, without introducing major changes in their crystallizability, integrity, or biological activity.

Acknowledgements

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