Brigitte Wittmann-Liebold (Ed.)

Methods in Protein Sequence Analysis

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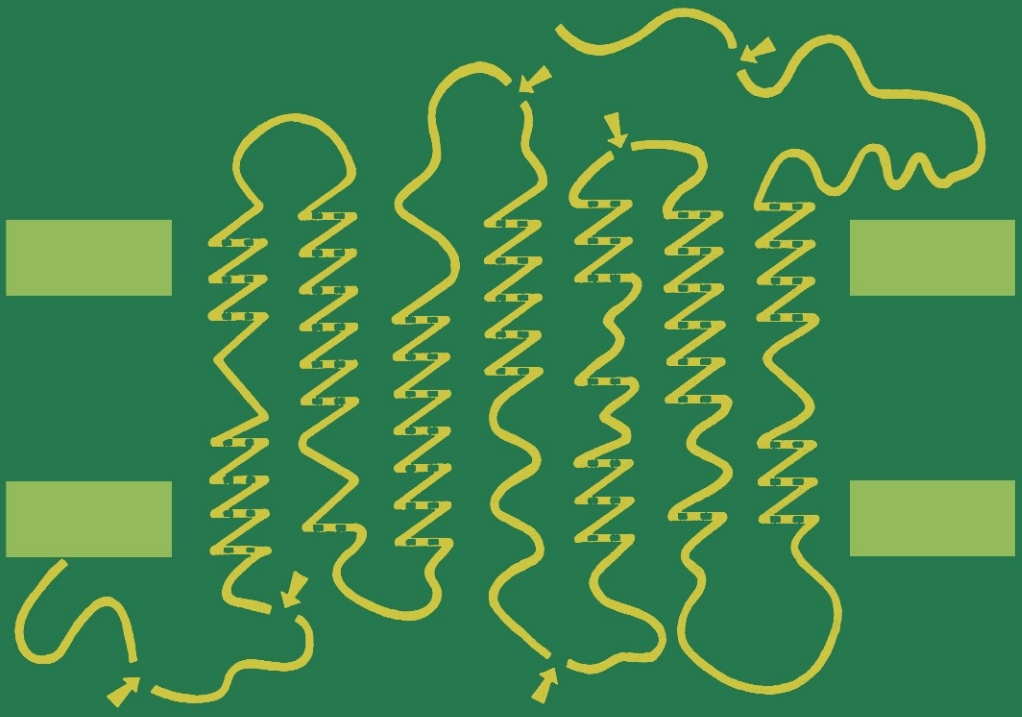

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9.5 Structural Studies on Crystals of Ribosomal Particles

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INTRODUCTION

Ribosomes, the universal cell organelles on which protein biosynthesis takes place, consist of two subunits which associate upon initiation of the biosynthetic process. Each ribosomal subunit is a defined assembly of proteins and ribonucleic acid chains. A typical bacterial ribosome, e.g. that of Escherichia coli, has a molecular weight of 2.300.000 Dalton. Its large (50S) subunit is of 1.450.000 Dalton and consists of 33 different proteins and 2 RNA chains. The small subunit (30S) is of 850.000 Dalton and contains 21 proteins and 1 RNA chain (Wittmann, 1982).

As objects for crystallographic studies the ribosomes are flexible, instable, asymmetric and of enormous size. However, systematic exploration of the crystallization conditions and development of innovative experimental techniques for fine control of the crystallization process, combined with sophisticated seeding, led to reproducible production of ordered three-dimensional crystals and two-dimensional crystalline arrays of whole ribosomes and of large and small ribosomal subunits (Table I and Yonath et al., 1986b, 1988; Makowski et al., 1987; Glotz et al., 1987; Trakhanov et al., 1987). In addition, crystals were obtained from large ribosomal subunits to which a nascent polypeptide chain and a tRNA molecule are attached (Gewitz et al., 1988), from mutated 50S subunits which lack one ribosomal protein (Yonath et al., 1986a) and of reconstituted subunits to which an undecagold or a tetrairidium cluster is covalently bound.

Using intense synchrotron X-ray beam, the best crystals, namely those from the 50S subunits of Halobacterium marismortui, diffract to 4.5 Å resolution (Makowski et al., 1987). In fact, there are indications for higher internal order: oriented arcs and distinct spots extending to 3.5 Å resolution, with spacings similar to those measured from gels of ribosomes have occasionally been detected on diffraction patterns of single crystals and on samples containing large numbers of microcrystals.

Inactive ribosomal particles could not be crystallized. Moreover, in all cases the crystalline material retained its integrity and biological activity for long periods in spite of the natural tendency of ribosomes to disintegrate and in contrast to the short life time of isolated ribosomes in solution.

Alongside with the growth of three-dimensional crystals, we produced ordered two-dimensional crystalline arrays of 30S subunits from Thermus thermophilus, of 50S subunits and 70S ribosomes from Bacillus stearothermophilus as well as of 50S particles to which a nascent protein and a tRNA molecule are attached. Some of these arrays were suitable for three-dimensional image reconstruction studies which led to models of the entire ribosome and of the large subunis (Yonath et al., 1987; Arad et al., 1987).

Crystallographic Studies

All crystals of ribosomal particles have large unit cells, display large mosaic spread (2.5-3 degrees) and are weakly diffracting. In all cases, the reflections with the highest resolution which were observed when fresh crystals were studied at ambient temperature, decayed within a few minutes of irradiation. Hence, over 260 crystals, aligned only visually at random orientations, had to be used in order to obtain a presumably complete film data set which, due to restrictions in rotation angles, contained only partially recorded reflections in this data set.

However, at cryo-temperature (85-100 K), flash-cooled crystals hardly show radiation damage for days (Fig. 1). The precooling treatment includes embedding of the crystals in viscous solutions which mimic their growth medium and mounting them on thin glass spatulas built so that the crystals are held at preferred orientations. Thus, when an intense synchrotron beam was used, under favorable conditions a complete diffraction data set could be collected from a single crystal. The current state of data collection and the different crystal forms are shown in Table 1.

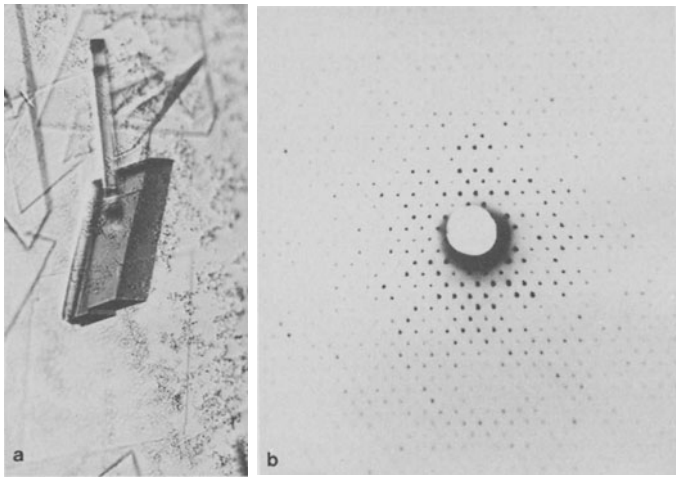

Fig. 1: (a) Crystals of 50S subunits from Halobacterium marismortui obtained as described by Yonath et al. (1988). (b) A 1° rotation pattern of (a) obtained at 85 K with synchrotron radiation.

Table 1. TWO- AND THREE-DIMENSIONAL CRYSTALS OF RIBOSOMAL PARTICLES

Source	Crystal Form	Cell Dimensions, (Å) determined by electron microscopy	Cell Dimensions, (Å) determined by X-ray crystallography	Resolution	Comments
70S E.coli	A*	340x340x590; 120°; $P6_3$			
70S B.st.	2D*,AS*	190x420; 91°; P2		40Å	
30S T.t.	M*		407x407x170; $P42_12$	9.9Å	N,H
50S H.m.	1, P*	310x350; 105°			
	2, P	148x186; 95°	147x181; 97°	13Å	
	3+,P	170x180; 75°	210x300x581; $C222_1$	5.5Å	N,H
50S B.st.	1, A	130x254; 95°			
	2, A	156x288; 97°			
	3, A	260x288; 105°			
	4, A	405x405x256; 120°			
	5, A	213x235x315; 120°			
	6+#, A	330x670x850; 90°	360x680x920; $P2_12_12$	18Å	N
	7+#, P		294x542x712; F222	11Å	N,H
	2D, A	145x311; 89°; P2		35Å	
	2D, AS	148x360; 109°; P1		25Å	

* All forms are of three-dimensionl crystals, unless marked by 2D.
A,M,P,AS=crystals were grown by vapor diffusion from low molecular weight alcohols (A), MPD (M), polyethylene glycol (P), or ammonium sulphate (AS).

N,H=crystallographic data collected from (N)native and (H) derivated crystals.

Same form and parameters for crystals of large ribosomal subunits of a mutant (-L11 protein) of the same source.

+ Same form and parameters for crystals of a complex of 50S subunits+tRNA molecule and a segment (18-20 mers) of a nacsent polypeptide chain.

B.st = Bacillus stearothermophilus, T.t. = Thermus thermophilus
H.m. = Halobacterium marismortoui

Phase Determination

Heavy-atom derivatization of an object as large as ribosomal particles requires the use of extremely dense and ultra-heavy compounds. We used an undecagold cluster (molecular weight 6200 Dalton) in which the gold core has a diameter of 8.2 Å and a tetrairidium cluster (m.w. 2300 Dalton) with an internal diameter of about 5.2 Å. Several variations of these clusters, modified with different ligands have been prepared. The cluster compounds in which all the moieties are amine or alcohol, are soluble in our crystallization and stabilizing solutions. Thus, they could be used for soaking, and indeed crystallographic data (to 18 Å resolution) collected from crystals of large ribosomal subunits from H. marismortui show isomorphous unit cell constants with observable differences in the intensities.

Because the surfaces of ribosomal particles have a variety of potential binding sites for such clusters, we also attempted to covalently bind heavy atoms to a few specific sites on the ribosomal particles prior to the crystallization. Alternatively, such clusters could be bound to natural or tailor-made carriers which bind specifically to ribosomes.

For direct binding to the surfaces of the ribosomes, the following approaches were taken: Firstly, free sulfhydryls on the surface of the 50S subunit have been identified by reacting with radioactive N-ethylmaleimide. The labeled proteins were identified by locating radioactivity in two-dimensional gels. It was found that in the case of 50S subunits from B. stearothermophilus there are mainly two proteins (BL11 and BL13) which bind N-ethylmaleimide. For H. marismortui a significant portion of the radioactivity was associated with a single protein. Secondly, the gold cluster mentioned above was prepared in such a way that it could be bound to accessible -SH groups.

Since this cluster is rather bulky, its accessibility was increased by the insertion of spacers, differing in length, to the cluster as well as to the free -SH groups on the ribosomal particles. It was found that a spacer of minimum length of about 10 Å between the -SH group of a ribosomal protein and the N-atom on the cluster is needed for significant binding. Preliminary experiments indicate that the derivatizated 50S particles can be crystallized.

The clusters may also be attached to isolated ribosomal components which can subsequently be incorporated into particles lacking these particular components. Thus, a mutant of B. stearothermophilus which lacks protein BL11 was obtained by growing cells in the presence of thiostrepton. The mutated 50S ribosomal subunits crystallized isomorphously with those of the wild-type. This indicates that BL11, the missing protein, is not involved in crystal forces in the native crystals. Furthermore, protein BL11 has only one sulfhydryl group, and binding of N-ethylmaleimide as well as the gold and the iridium clusters to it does not prevent its reconstitution into the mutated particles and does not inhibit the biological activity and crystallizability of the particles. Two-dimensional gel electrophoresis of the reconstituted 50S particles to which a heavy-atom cluster was bound, as well as neutron activation analysis

show that each subunit contains one cluster. Furthermore, using the neutron activation method, we could show that the cluster is bound to the 50S subunits even within the crystals. Crystallographic data have already been collected from these crystals.

Three-Dimensional Image Reconstruction

The large size of ribosomal particles which is an obstacle for crystallographic studies permits direct investigation by electron microscopy. Investigation by electron microscopy of crystalline ribosomal particles may show the location and orientation of the particles within the crystals. A model obtained by three-dimensional image reconstruction of two-dimensional sheets may also be used for gradual phasing of low resolution crystallographic data. To this end, we have initiated three-dimensional image reconstruction studies.

Well-ordered two-dimensional sheets of 70S ribosomes and of 50S ribosomal subunits from B. stearothermophilus have been subjected to three-dimensional image reconstruction studies at 47 Å and 30 Å resolution, respectively (Yonath et al., 1987; Arad et al., 1987). In both cases the reconstructed particles have average dimensions similar to those determined by other physical methods (Wittmann, 1983). On the basis of the known molecular weight of these particles and of the volume obtained from the three-dimensional image reconstruction, the calculated densities are in accord with values calculated for the crystals of the 50S subunits from H. marismortui and other nucleoproteins (Makowski et al. 1987).

Two-dimensional sheets of 70S particles from B. stearothermophilus are built of dimers and packed in relatively small unit cells (about 192 x 420 Å). Analysis of the models obtained by three-dimensional reconstruction of sheets stained with gold-thioglucose (Fig. 2) show that the two ribosomal subunits are arranged around an empty space, large enough to accommodate components of protein biosynthesis, such as the tRNA molecules and the elongation factors (Arad et al., 1987).

We have assigned a portion of the reconstructed 70S particle as the large subunit by correlating it to the image of this subunit which was reconstructed by us from two-dimensional sheets (Fig. 2 and in Yonath et al., 1987). The model of the 50S subunits shows several projecting arms, two of which are longer than the others, arranged radially around a narrow elongated cleft which is formed between the projecting arms and turns into a tunnel of a diameter of up to 25 Å and 100 – 120 Å length (Fig. 2). A similar feature was also detected on ribosomes from chick embryos by three-dimensional image reconstruction (Milligan and Unwin, 1986).

The functional significance of the tunnel is still to be determined. However, originating at the presumed site of actual protein biosynthesis and terminating on the other end of the particle, and being of a diameter large enough to accommodate a peptide of around 40 amino acids in any conformation, this tunnel appears to provide the path taken by the nascent polypeptide chain. Protection by the ribosome of newly formed

peptides of up to 40 amino acids from proteolytic enzymes, was observed earlier by biochemical methods (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Smith et al., 1978). It remains to be seen whether the tunnel terminates at a location compatible with that assigned by immune electron microscopy as the exit site for the growing polypeptide chain (Bernabeau and Lake, 1982).

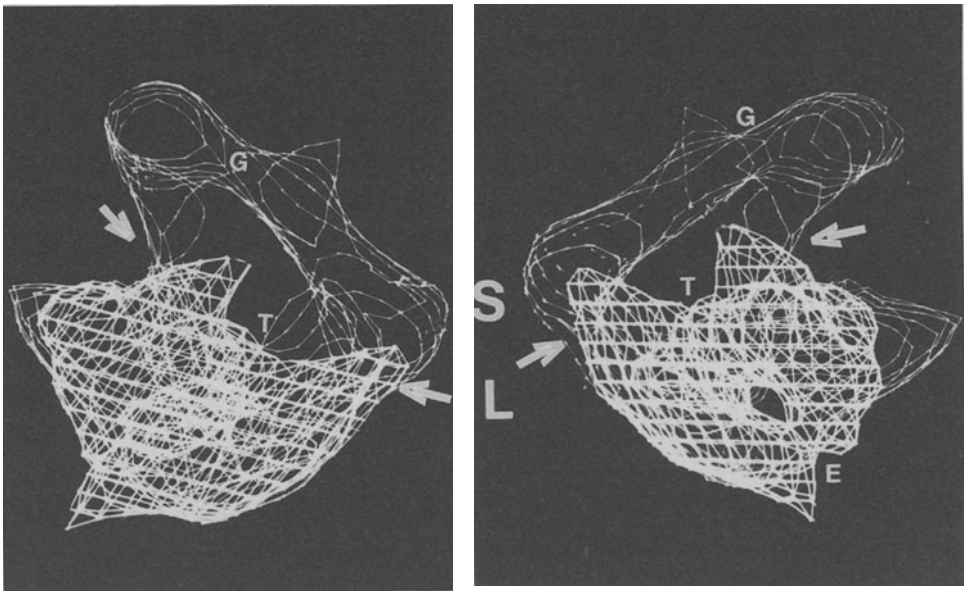

Fig. 2: Superposition of computer graphic display of the outline of the reconstructed models of the 70S ribosome (in lines) and of the 50S ribosomal subunits (drawn as a net). L and S indicate the 50S and the 30S subunits, respectively. The arrows point at the interface between the two subunits. G points at a groove, rich in RNA in the small subunit. Depicted in the 50S subunits are (T) which shows the cleft and the entrance to the tunnel and (E) which shows the presumed site for the exit of the nascent chain.

Since the 70S particles used for these studies were harvested while being active in protein biosynthesis, it is feasible that nascent protein chains were attached to them. This may be the reason why in this case the tunnel is only partially resolved.

We assumed that the part of the 70S ribosome which was not assigned as the large subunit represents the small one (Fig. 2). There is a similarity between the model of the small subunit obtained by visualization of single particles (Wittmann, 1983) and that revealed by our studies. However, isolated 30S particles appear somewhat wider than those reconstructed within the 70S particles. This may be a consequence of the contact of the isolated particles with the flat electron microscope grid. In contrast, particles within the crystalline sheets are held together by a network of crystalline forces. These may stabilize the conformation of the particles and diminish or even eliminate the influence of the flatness of the grids.

Comparison between the reconstructed model from sheets stained with uranyl acetate (acting also as a positive stain which may interact with phosphates) and those stained by gold-thioglucose (an inert stain) indicated the regions where there is a high concentration of RNA. Such regions are located at a narrow groove on the body of the small subunit (at about a third of its total length) and at the interface of the small and the large subunits, in good agreement with other studies (Milligan and Unwin, 1986; Brimacombe et al., 1988).

Concluding Remarks

We have demonstrated here that crystallographic studies may be carried out on intact ribosomal particles. We expect that these studies supported by electron microscopy and combined with the available biophysical, biochemical and genetic knowledge, will yield a reliable model for the ribosome and contribute to the understanding of the mechanism of protein biosynthesis at the molecular level.

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REFERENCES

Arad T, Piefke J, Weinstein S, Gewitz HS, Yonath A, Wittmann H.G. (1987) Three-dimensional image reconstruction from ordered arrays of 70S ribosomes. Biochimie 69:1001-1006

Bernabeau C, Lake JA (1982) Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit: Immune mapping of the nascent chain. Proc Nat Acad Sci USA 79:3111-3115

Blobel G, Sabatini DD (1970) Controlled proteolysis of nascent polypeptides in rat liver cell fractions. J Cell Biol 45:130-145

Brimacombe R, Atmadja J, Stiege W, Schüler D (1988) A detailed model of the three-dimensional structure of Escherichia coli 16S ribosomal RNA in situ in the 30S subunit. J Mol Biol 199:115–136

Gewitz HS, Glotz C, Piefke J, Yonath A, Wittmann HG (1988) Two-dimensional crystalline sheets of Bacillus stearothermophilus 50S ribosomal subunits containing a nascent polypeptide chain. Biochimie 70:645–648

Glotz C, Müssig J, Gewitz HS, Makowski I, Arad T, Yonath A, Wittmann HG (1987) Three-dimensional crystals of ribosomes and their subunits from eu- and archaebacteria. Biochem Internat 15:887–895

Makowski I, Frolow F, Saper MA, Wittmann HG, Yonath A (1987) Single crystals of large ribosomal particles from Halobacterium marismortui diffract to 6 Å. J Mol Biol 193:819–821

Malkin LI, Rich A (1967) Partial resistance of nascent polypeptide chains to proteolytic digestion due to ribosomal shielding. J Mol Biol 26:329–346

Milligan RA, Unwin PNT (1986) Location of exit channel for nascent protein in 80S ribosomes. Nature 319:693–696

Smith WP, Tai PC, Davis BD (1978) Interaction of secreted nascent chains with surrounding membrane in Bacillus subtilis. Proc Natl Acad Sci USA 75:5922–5925

Trakhanov SD, Yusupov MM, Agalarov SC, Garber MB, Ryazantsev SN, Tischenko SV, Shirokov VA (1987) Crystallization of 70S ribosomes and 30S ribosomal subunits from Thermus thermophilus. FEBS Lett 220:319–322

Wittmann HG (1982) Components of bacterial ribosomes. Annual Rev Biochem 51:155–183

Wittmann HG (1983) Architecture of prokaryotic ribosomes. Annual Rev Biochem 52:35–65

Yonath A, Frolow F, Shoham M, Müssig J, Makowski I, Glotz C., Jahn W, Weinstein S, Wittmann HG (1988) Crystallography of ribosomal particles. J Cryst Growth 90:231–244

Yonath A, Leonard KR, Weinstein S, Wittmann HG (1987) Approaches to the determination of the three-dimensional architecture of ribosomal particles. Cold Spring Harbor Symposium 52:729–741

Yonath A, Leonard KR, Wittmann HG (1987) A tunnel in the large ribosomal subunit revealed by three-dimensional image reconstruction. Science 236:813–816

Yonath A, Saper MA, Frolow F, Makowski I, Wittmann HG (1986a) Characterization of single crystals of the large ribosomal particles from a mutant of Bacillus stearothermophilus. J Mol Biol 192:161–162

Yonath A, Saper MA, Makowski I, Müssig J, Piefke J, Bartunik HD, Bartels KS, Wittmann HG (1986b) Characteristics of single crystals of the large ribosomal subunits from Bacillus stearothermophilus. J Mol Biol 187:633–636