TOWARDS ATOMIC RESOLUTION OF PROKARYOTIC RIBOSOMES: CRYSTALLOGRAPHIC, GENETIC AND BIOCHEMICAL STUDIES

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1. INTRODUCTION

The studies reported here were initiated and inspired by the late Prof. H.G. Wittmann. From the early stages of this project, when it was widely believed that even the initial steps in determining the molecular structure of ribosomes are impossible, until his last days, Prof. Wittmann was actively involved in the experimental design and in the actual studies. We have no doubt that without his motivation, optimism, guidance and support, this project would not have reached its current stage.

In this chapter we describe the progress since the last "Ribosome Meeting" (summarized by Hill et al., 1990). Of particular significance are: the growth of improved ribosomal crystals, which diffract to almost atomic resolution; the collection and the evaluation of X-ray and neutron crystallographic data of high quality; the combination of metallo organic biochemistry, genetic manipulations and functional studies, which led to quantitative labeling of ribosomal crystals with an improved form of a multi-heavy-atom cluster (undecagold); the crystallization of thermophilic ribosomes trapped in defined functional states; the quantitative binding of the undecagold cluster to $tRNA^{Phe}$ in a fashion which does not hamper its recognition by its synthetase and its binding to ribosomal particles; the preparation and crystallization of core ribosomal particles lacking one or a few selected proteins; the progress in amino acid sequencing of the halophilic and thermophilic ribosomal proteins; the genetic insertion of -SH reactive groups to the surface of the

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halophilic ribosome; the hybridization of cDNA oligomers, which are complementary to exposed single strands of rRNA of halophilic and thermophilic ribosomes; the purification and characterization of a ribonucleoprotein complex from halophilic ribosomes; the growth of highly ordered two-dimensional arrays of ribosomal particles, which may be investigated by electron microscopy without staining; and new suggestions for alternative assignments of functional sites in the electron-microscopical reconstructed models.

2. CRYSTALLOGRAPHY

I. Crystals of Ribosomal Particles Diffract to 2.9 Å Resolution

The most striking recent achievement is the growth of crystals of 50S subunits from *Haloarcula marismortui*, which have a low mosaic spread and improved mechanic strength, yielding usable data to almost atomic resolution (Fig. 1). This high internal order was most unexpected crystallographically and in view of the apparent sequence heterogeneity, in the two genes encoding the rRNA of this subunit (Mylvaganam and Dennis, 1992). These crystals grow in solutions mimicking the halophilic intracellular environment in the presence of a few mM Cd^{++} (von Böhlen et al., 1991). The exceptional effect of the Cd^{++} is noteworthy, as crystals grown under the same conditions but without it diffracted nominally to 4.5 Å, but due to their high mosaicity and tendency for fragmentation, their X-ray crystallographic data were useful to only 7-9 Å resolution.
Considerable difficulties were encountered in efforts to crystallize halophilic 70S ribosomes, most probably due to their marked tendency to dissociate. To avoid dissociation the halophilic 70S ribosomes were kept at 1.9 M ammonium sulfate. Initial indications for the growth of microcrystals of the halophilic 70S ribosomes were obtained by investigating crystallization mixtures in the electron microscope. In addition, crystals of reasonable size were recently grown from halophilic 30S subunits.

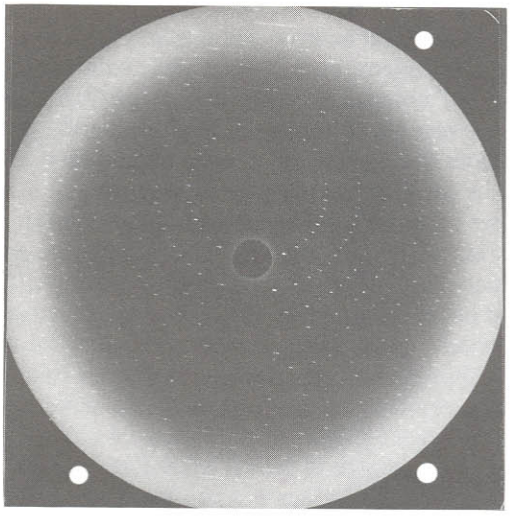

Figure 1. A rotation photograph of a crystal of 50S subunits from *H. marismortui* grown as described in von Böhlen et al., 1991. The pattern was obtained at 90 K at Station F1/CHESS, operating at 5.3 Gev and 50 mA. Crystal to film distance=220 mm, collimator=0.1 mm; wave length = 0.9091 Å.

II. Specific Concerns in Data Collection and Evaluation

Due to the weak diffracting power and the large unit cells of the crystalline ribosomal particles (TABLE I), virtually all the crystallographic studies have to be performed with intense synchrotron radiation. To eliminate the extreme radiation damage of these crystals, data are collected at cryogenic temperatures (about -180°C) from shock frozen crystals. Under these conditions, the ribosomal crystals diffract with no observable decay for periods longer than needed for collecting a full set of data (typically a few days), and the irradiated crystals can be stored for months. To facilitate cryogenic data collection we designed experimental procedures which accommodate the special features of the ribosomal crystals, i.e. fragility, sensitivity, thin edges, etc. (Berkovitch-Yellin et al., 1993). Data are collected using the screenless rotation method. The weak diffraction power of ribosomal crystals dictates extremely long exposure times (8-24 min./deg.), resulting in high background levels, which limit the maximum oscillation range. On the other hand, due to the mosaicity, which even for our best crystals is still significant (0.3-1.0°), large rotation ranges are desirable for obtaining a sufficient number of fully recorded reflections, required for internal scaling. A third factor influencing the data collection strategy is the extremely steep falloff of intensities as a function of resolution. Thus, under conditions optimized for collecting the high resolution reflections, which are the weakest, the low-resolution reflections are overexposed, even when using Imaging Plates, which have a large dynamic range. These contradictory requirements dictate data collection in several resolution shells, a procedure which became feasible only after introducing data collection at cryogenic temperature.

TABLE I. PARAMETERS OF RIBOSOMAL CRYSTALS

Source	Grown From*	Cell Dimensions (Å)	Resolution (Å)
70S T.t.	MPD	524x524x306; $P4_12_12$	20
70S T.t. complexed +	MPD	524x524x306; $P4_12_12$	15
30S T.t	MPD	407x407x170; $P42_12$	7.3
50S H.m.°	PEG	210x300x581; $C222_1$	2.9
50S T.t.	AS	495x495x196; $P4_12_12$	15
50S B.st.^	A	360x680x920;$P2_12_12$	18
50S B.st.^#	PEG	300x547x384;114°; C2	11

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

* crystals were grown by vapor diffusion in hanging drops from solutions containing methyl-pentane-diol (MPD), polyethyleneglycol (PEG), ammonium sulfate (AS) or low molecular weight alcohols (A). For exact conditions see (Berkovitch-Yellin et al., 1992).

Reported 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 of 70S ribosomes, two molecules of phetRNA^{phe} and an oligomer of 35 uridines.

° Same form and parameters for crystals of 50S lacking protein HmaL11.

^ Same form and parameters for crystals of 50S lacking protein BL11 and for modified particles with an undecagold-cluster.

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

The high resolution diffraction data sets contain hundreds of thousands reflections. For example, 1,434,786 reflections were measured for the shell 10-3.5 Å from crystals of the halophilic 50S subunits, yielding 145,249 unique intensities. It was found that the evaluation of the ribosomal data is still not a routine task, although some special computational tools have been developed specifically for this purpose. Despite these difficulties, the evaluated data are of quality comparable to that obtained from crystalline proteins of average size. Thus, for above 50% completeness, the typical values for R-merge (I) at relatively high resolutions (6 Å) are in the range of 5-10% (Berkovitch-Yellin et al., 1993).

3. PHASING STRATEGIES

The phasing of X-ray amplitudes which enables calculation of electron density maps remains the most difficult part in crystal structure determination. The classical methods for phasing are MIR and SIR (Multiple and Single Isomorphous Replacement) which require the specific binding of heavy atoms at limited number of sites within the unit cell. Both methods are based on differences in intensities of the reflections of native and derivatized crystals, thus are dependent on close to ideal isomorphism. Due to the large size and the internal flexibility of ribosomal particles, achieving ideal isomorphism is doubtful. In some instances there is an apparent isomorphism. In others, a significant nonisomorphism 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. Whether apparent or real, the lack of isomorphism complicates phasing by difference methods. However, successive exposures at different wave lengths of crystals derivatized by compounds with a significant anomalous scattering component may facilitate phasing by MAD (Multi-wavelength Anomalous Diffraction). Attempts in this direction are underway.

I. Preparation of Heavy Atom Derivatives: an Improved Gold Cluster

Heavy atom derivatives are obtained by introducing electron-dense compounds to the crystalline lattice at distinct locations. Conventional derivatization is achieved either by soaking the crystals in solutions of, or by co-crystallization with electron-dense compounds. For proteins of average size, useful isomorphous derivatives consist of one or a few heavy-metal atoms. For ribosomal crystals much heavier compounds are required. To reach the required electron-density and to achieve quantitative and site specific derivatization, we developed a monofunctional gold cluster (GC, m.w.=6200 Da), with a core of 11 gold atoms linked directly to each other (Weinstein et al., 1989). Simulation studies have indicated the potential phasing power of this cluster.

Maleimido derivative **Iodoacetamide derivative**

Figure 2. The new short arms of the gold cluster

The first targets for binding the cluster to the ribosomes were exposed free sulfhydryls. To enable the binding of the gold cluster, we attached to it a short chemically reactive arm of about 3 to 4 Å in length (Fig. 2) with either a maleimido or iodoacetyl moieties at its end. The latter was designed to avoid the chirality introduced by the reaction with the double bond of the maleimido moiety. The improved cluster was quantitatively bound to base 47 of $tRNA^{phe}$ of E. coli (see 5.I) and to natural or genetically engineered sulfhydryl groups of isolated ribosomal proteins, which should subsequently be incorporated into core particles lacking them, in a fashion similar to that reported previously (Weinstein et al., 1989, 1992; Yonath et al., 1990).

II. Crystals of Halophilic Ribosomal Cores, Lacking Selected Proteins

Four ribosomal proteins were selectively detached from the 50S subunits from *H. marismortui* by dioxane. This approach was chosen due to the resistance of archaebacteria, to most antibiotics used for mutant selection (Amils et al., 1990). All the removed proteins were fully reconstituted. One of them, HmaL11, binds reagents specific to -SH moieties, but it was found that once its cysteine is modified, the protein could not be incorporated into core particles. This way we obtained 50S subunits, lacking protein HmaL11 which crystallize under the conditions used for the native subunits, and diffract to 10 Å resolution. We also crystallized 50S particles from which proteins HmaL1, HmaL11 and HmaL12 were removed by chemical methods.
The crystals of the depleted particles may be useful for locating the sites of the missing proteins at low resolution, thus providing an anchor for initial interpretation (see 6).

III. Inserting exposed sulfhydryls into the surface of the halophilic ribosomes

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                  11                                    33
1 AGTIEVLVPG  G EANPGPPLG PELGPTPVDV QA V VQEINDQ TAAFDGTEVP VTVKYDDDGS
              C                                     C

61 FEIEVGVPPT AELIKDEAGF ETGSGEPQED FVADLSVDQV KQIAEQKHPD LLSYDLTNAA

                127                   142
121 KEVVGT  C TSL GVTIEGENPR E F KERIDAGE YDDVFAAEAQ  A
            S                 C
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mutated HmaL11 protein	(wt)	(1)	(2)	(3)	(4)
reconstitution into minus L11 ribosomes	+	+	+	+	+
binding of NEM to the isolated protein	+	-	+	+	+
reconstitution of NEM-labeled protein into minus L11 ribosomes	-	-	-	-	+

Figure 3. Genetic insertions of cysteines into protein HmaL11. Amino acid sequence of protein HmaL11. Four different mutant proteins were produced, namely (1) HmaL11mut11, (2)HmaL11mut11/127, (3) HmaL11mut11/127/142 and (4) HmaL11mut11/33/127. The binding of NEM to the different proteins and its reconstitution into ribosomes lacking L11 was tested.

As the halophilic r-proteins HmaL1 HmaL11 and HmaL12, can be removed from the core particles and as their genes have been cloned (Arndt and Weigel, 1990), the insertion of -SH groups into the surface of the ribosome became feasible. Several mutants were produced by oligonucleotides directed in-vitro mutagenesis. The cys codon of the wild type HmaL11 was exchanged to a ser codon, and codons specific for cys were inserted into different positions. A similar procedure was applied to HmaL1, except for the necessity to eliminate natural cysteine, since HmaL1 does not contain any. The mutated genes were cloned into the vector (pet11d) and the mutated proteins were overexpressed in E. coli. These mutated, overexpressed proteins could be reconstituted into ribosomal cores lacking them. Screening of the suitability of the mutated proteins for binding heavy atom clusters, is in progress, and indications for the incorporation of protein HmaL11 in which amino acid 34 is a cysteine, into core 50S particles, have been obtained (Fig. 3).

For further insertions of exposed cysteines in other halophilic r-proteins we exploit the advanced stage of genetic sequencing (see 6.III), the determination of conditions for reconstitution of halophilic ribosomes (Sanchez et al., 1990), the mapping of the surface of the halophilic ribosome by limited proteolysis (Kruft and Wittmann-Liebold, 1991) and by the determination of exposed -SH groups as a function of the ionic strength (Weinstein et al., 1989; Sagi and Weinstein, to be published).

IV. Other Phasing Methods

The molecular replacement method is based on the search for best positioning of a model of the investigated compound in the crystallographic unit cell. The low-resolution models of 70S and 50S ribosomal particles from *B. stearothermophilus* (Sec. 4 and in Yonath et al., 1987; Arad et al., 1987; Berkovitch-Yellin et al., 1990), are being employed for these searches (Eisenstein et al., 1991), together with X-ray crystallographic data collected from three-dimensional crystals of ribosomal particles from *T. thermophilus* and *H. marismortui*. The use of reconstructed images of ribosomal particles of one bacterium with data obtained from crystals of ribosomes from another one, is based on the assumption that at the resolution limits of the reconstructions the gross structural features of prokaryotic ribosomes are similar. Parallel phasing attempts, exploiting maximum entropy, gas condensation and other computational methods for phasing, have also yielded encouraging results (N. Volkmann, M. Roth, E. Pebay-Peroula, S. Subbiah, M. Eisenstein, D. Rabinovitch, Z. Berkovitch-Yellin, A. Zaytzev-Bashan, unpublished).

4. ELECTRON MICROSCOPY AND IMAGE RECONSTRUCTION

In 1987 we reconstructed models for the 50S subunit and 70S ribosomes at 28-47 Å, using electron micrographs of tilt series of two-dimensional sheets (Yonath et al., 1987: Arad et al., 1987). Despite their low resolution, we detected in these models several key features, most of which are associated with internal vacant spaces or partially filled hollows, not observed earlier in prokaryotic ribosomes. The significant similarities in specific features in the reconstructed models were used to assess their reliability and to tentatively assign biological functions to some structural features.

I. The Original Interpretation of the Reconstructed Models

The existence of an internal tunnel in large ribosomal subunits was suggested more than two decades ago and further substantiated recently, as a result of several biochemical

experiments which showed that the ribosome masks the newly synthesized protein chains (for review see Yonath and Wittmann, 1989; Yonath et al., 1990; Berkovitch-Yellin et al., 1990). So far this tunnel was revealed only by diffraction studies. It was first observed as a narrow elongated region of low density in images reconstructed at very low resolution from 80S ribosomes of chick-embryos (Milligan and Unwin, 1986). As our image reconstruction studies were performed at somewhat higher resolution (28 Å), they resulted in a more precise description, albeit still suffering from the inherent shortcomings of electron microscopy at ambient temperature (Yonath et al., 1987; Berkovitch-Yellin et al., 1992). An almost identical model, including an internal tunnel, was recently reconstructed from two-dimensional sheets of 50S particles from *T. thermophilus*, obtained on lipid mono-layers, and investigated at cryo-temperature (Yoshinori Fujiyoshi, personal communication). In addition, a low density region was observed in features seen in the density map obtained from three-dimensional crystals of halophilic 50S subunits at 30 Å resolution, using neutron diffraction data, phased by direct methods (Eisenstein et al., 1991).

Image reconstructions using diffraction data from several tilt series of two-dimensional sheets of 70S ribosomes gave rise to a few models, sharing several common features (Yonath and Wittmann, 1989, Yonath et al., 1990; Berkovitch-Yellin et al., 1990). The two most deviating ones differ mainly in their bulkiness (called below "thick" and "thin"). However both contain a few rather long tunnels. The most striking similarity between the two models is in their internal void, which accounts for about 20% of their volume. Steric considerations showed that this void is spacious enough to accommodate up to three tRNA molecules together with other non-ribosomal components participating in protein biosynthesis. Therefore, it is conceivable that this void is the location for the various enzymatic activities of protein biosynthesis.

As we had no reason to prefer one reconstruction on the other, we first focused on the "thin" one, since it is more detailed and therefore more restrictive. Interestingly, our original assignments in the "thin" model fit well also the "thick" one. Part of the 70S particle was tentatively identified as the large subunit, based on a visual and computational fit of the overall shapes as well as on the directionality of the longest tunnel. A distinct region rich in rRNA was revealed (by uranyl-acetate staining) within the part of the 70S ribosome left for the 30S subunit (Fig. 4 and in Yonath and Wittmann, 1989, Berkovitch-Yellin et al., 1990; Yonath et al., 1990). Similarly, a region of a high stain-density was detected by electron microscopical investigations of isolated 30S subunits (Oakes et al., 1990). As it is known that the ribosome masks stretches of 30-40 nucleotides of mRNA molecules (Kang and Cantor, 1985), we assigned as the mRNA path, a groove within this region compatible with the biochemical findings.

In a model-building experiment, a molecule of tRNA was placed in the intersubunit space with its anticodon close to the tentative mRNA binding site and its CCA-terminus positioned so that the peptidyl group could extend into the tunnel (Fig. 4). In this orientation the tRNA molecule may form a variety of interactions with the walls of the intersubunit space. It should be mentioned that at the current resolution the two crystallographically determined conformations of tRNA (for review see Moras, 1989) can be placed in the intersubunit space in a very similar manner.

II. Alternative Interpretations of the Reconstructed Models

Although we do not doubt the feasibility of our original interpretation, we have been stimulated to consider tentative assignments in a model-building exercise. Recently a model for the 70S ribosome from *E. coli* was reconstructed by averaging the electron microscopical shapes of a large number of single particles embedded in vitrified ice (Frank et al., 1991).

Despite the different technique and the use of ribosomes from a different source, this reconstruction led to a model almost identical in shape to our "thick" 70S model. Since this new reconstruction is based on investigation of unstained samples, the contrast between regions rich in rRNA and the rest of the particle could be observed. The assignments based on this information (Frank et al. 1991) are consistent with our suggestion that the intersubunit void provides the site of the peptidyl transferase reaction (Arad et al, 1987; Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990; Yonath et al., 1990; Hansen et al., 1990).

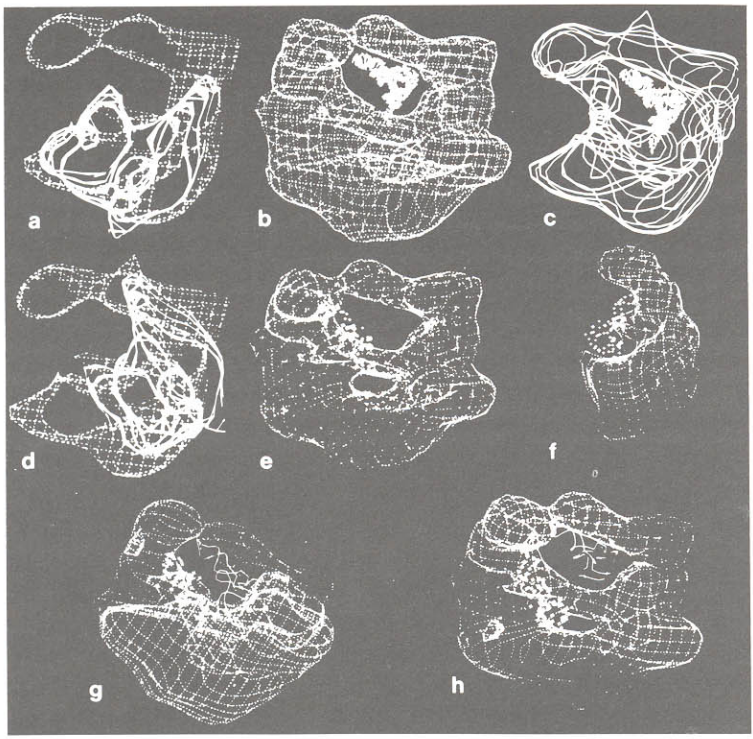

Figure 4. Functional assignments in reconstructed 70S ribosomes.

Top panel: The original assignments, based on the best fit in the direction of the long tunnel and in the shape of the 50S subunit and the part assigned to it in the "thin" reconstructed model of the 70S ribosome; (a) A slice of 50 Å in depth of the superposition of the reconstructed models of the "thin" 70S ribosome (shown as a net) and the 50S subunit (in full lines); (b) and (c) tRNA molecules, model built into the intersubunit free space in the "thick" and the "thin" models of 70S, respectively, with its CCA end pointing into the tunnel.

Middle Panel: The alternative assignments, based on the best fit in the direction of the shorter tunnel and on the distribution of mass within the ribosome (Frank et al, 1991); (d) A slice of 50 Å in depth of the superposition of the reconstructed models of the "thin" 70S ribosome (shown as a net) and the 50S subunit (in full lines); (e and f) Two orthogonal views of the reconstructed "thick" model of the 70S ribosome. A tRNA molecule is "Model built" into the intersubunit free space, with its CCA end pointing into the tunnel.

Bottom Panel: (g) and (h) Two views of the "thick" 70S ribosome. The intersubunit free space of each model contains two model-built tRNA molecules, each representing a different assignment. That placed according to the original interpretation is shown as a continuous line. The second, placed according to the alternative interpretation, is shown as a chain of plus (+) signs.

To assess the compatibility of the recent assignment with our original one, we attempted to fit our reconstructed model of the 50S subunits into the "thin" and the "thick" 70S models. We found that positioning the large subunit in the "thin" 70S model at any location other than our original one, leaves hardly any density for accommodating the small subunit, whereas the "thick" model possesses sufficient density for the 30S subunit in almost any orientation of the 50S model. However, even the best alignment between the 50S and the "thick" ribosome, based on the direction of the shorter tunnel, suffers from rather poor fit between the shapes of the 50S and the part assigned to it on the 70S ribosome, leaving several extensive regions of unmatched density. Remarkably, the density assigned to the 30S subunit contains a groove, in a region poor in rRNA with dimensions suitable to host a stretch of 30-40 nucleotides of mRNA. Like the groove described in our original interpretation (Arad et al, 1987; Yonath and Wittmann, 1989; Berkovitch-Yellin et al., 1990), the newly assigned one is positioned so that a molecule of tRNA can bridge between it and the entrance to the tunnel in the 50S subunit (Fig. 4) while interacting with the walls of the intersubunit space. Interestingly, a third interpretation, which utilizes the intersubunit void for the peptidyl transferase was recently suggested (Lim et al., 1992).

The fact that our original suggestion to assign the intersubunit void as the center of the biosynthetic process became widely accepted is most satisfying, although it is clear that neither of the reconstructions is of sufficient resolution to provide an unambiguous assignment. Perhaps the main lesson from these model building exercises is that the intersubunit void is sufficiently large, so that its ability to accommodate the components of protein synthesis in a sterically reasonable arrangement does not critically depend on the assignment of the small and the large subunits. It is evident that a more detailed interpretation may be feasible only after the maturation of further diffraction studies at higher resolution. Attempts in this direction are currently underway. High quality ordered arrays of ribosomal particles, which diffract to 12-15 Å resolution and can be studied unstained in vitrified ice, have been recently obtained (W. Chiu, M. Schmidt, T. L. Guan, T. Arad, A. Yonath, J. Piefke and F. Franceschi, to be published).

5. FUNCTIONAL EXPERIMENTS INFLUENCING STRUCTURAL STUDIES

Despite the uncertainties in our assignments, the detection of internal features in the ribosome stimulated further functional and structural investigations. As a result it was shown that ribosomes mask nascent natural proteins more efficiently than artificial homo-polypeptides (Evers and Gewitz, 1989; Kolb et al., 1990) and that the latter may choose an exit path slightly different than that of naturally occurring proteins (Hardesty et al., 1990). It is conceivable that a common feature located at the amino termini of natural proteins (e.g. fMet for prokaryotes) has a role in guiding the nascent protein chain into the tunnel. A failure to enter the tunnel may influence the biosynthetic machinery at early stages and lead to the termination of the process. This hypothesis may explain why usually only 40-60% of well prepared ribosomes are active in in vitro production of relatively long polypeptides, although almost all ribosomes bind quantitatively mRNA and tRNA (Rheinberger and Nierhaus, 1990) and why short and long chains of newly synthesized polylysine or polyphenylalanine migrate in different modes (Hardesty et al., 1990).

I. Crystals of complexes of ribosomal particles in defined functional states

(a) Crystals of complexes of 50S subunits: To investigate the chemical properties of the exit path of nascent polypeptide chains, we obtained small three-dimensional crystals

and two-dimensional sheets of reasonable-size from 50S subunits of either *H. marismortui* or *B. stearothermophilus*, with a short polyphenylalanine or polylysine (8-18 amino acids) and one molecule of their cognate tRNA (Gewitz et al., 1988; Müssig et al., 1989). Such complexes may be used for illuminating the exit path of the nascent protein, as well as for derivatization, providing the possibility to label the nascent chain with heavy atoms (see 6).

(b) Crystals of complexes of 70S ribosomes: We assumed that the intersubunit space contributes to the motion of the ribosome, allowing for the dynamics involved in the biosynthetic process. At the same time it contributes to the poor internal order of the crystals of 70S ribosomes. To minimize the flexibility and to increase the homogeneity of the crystallized material, complexes composed of 70S ribosomes from *T. thermophilus*, with one or two Phe-tRNA^{phe} molecules and chains of about 9 or 35 uridyl residues were crystallized (Yusupova et al., 1991; Hansen et al., 1990). In this complexes the intersubunit space may have a limited motional freedom, as a result of trapping the ribosomes into relatively rigid conformations and the mRNA is of a length which may fit into the groove in the 30S subunit, so that no long stretches of it project into the solvent.

Crystallographic data collected from the crystals of the complex with oligo(U)-35-mer showed that indeed the crystals of the complex are superior to those of isolated ribosomes, despite its non optimal composition (e.g. using a homopolynucleotide rather than mRNA chains of a designed sequence). We observed a dramatic improvement in the reproducibility in crystal growth and in the internal order of the crystals. Thus, the crystals of the complex diffract to a resolution of 15 Å, compared with about 20 Å, obtained for the best crystals of 70S ribosomes (Hansen et al., 1990; Trackhanov et al., 1989; Berkovitch-Yellin et al., 1991). It is conceivable that better crystals may be obtained by using mRNA chains of defined lengths and sequences. Therefore we synthesized mRNA chains of a length which can be accommodated within the intersubunit space and a sequence suitable for productive binding to thermophilic ribosomes, with elements promoting initiation of protein biosynthesis. As little is known about the functional aspects of thermophilic ribosomes, we are currently engaged in their characterization. Attempts to produce and crystallize similar complexes of halophilic 70S ribosomes are underway, and the purification of several halophilic tRNA molecules from *H. marismortui* has been initiated (Safro et al., 1992).

(c) Quantitative labeling of tRNA: Since tRNA is an essential part of the crystalline functional complexes, it may be exploited as a carrier of heavy-atom clusters. Base 47 of *E. coli* tRNA^{phe} is a naturally modified uridine nucleoside (ACP3U), containing an exposed reactive primary amino group. Iminothiolane was used for converting this amino group into a reactive sulfhydryl, which was used, in turn, for binding the gold cluster with its short or long arms. The resulting modified tRNA (tRNA^{phe}-GC) was visualized by dark field scanning transmission electron microscopy at cryogenic temperature. Using radioactive tRNA^{phe}-GC (Boeckh and Wittmann, 1991), it was established that the modified tRNA molecule can be aminoacylated by its cognate synthetase and binds to 70S ribosomes and to 30S subunits from *T. thermophilus* with the same stoichiometry found for native tRNA^{phe}, in the presence or the absence of poly(U) (Weinstein et al., 1992).

II. DNA Oligomers, Complementary to Exposed rRNA

Exposed single-strand rRNA segments which may be complemented by DNA oligomers (e.g., Weller and Hill, 1991) are currently being located on halophilic and thermophilic

ribosomes. A variety of DNA oligomers, targeting naturally exposed rRNA regions as well as those which become exposed by removing selected ribosomal proteins, have been synthesized. These may be used for derivatization, as we prepared them with a thiol group at the 5' end and bound the gold cluster to it (J.Späthe, S.Hottenträger, B.Wittmann-Liebold and F.Franceschi, unpublished). The regions of rRNA which were targeted by us are: bases 1125-1158 of 23S RNA of the mutant lacking protein L11 from *B. stearothermophilus*, which, in the wild type are masked by protein L11; the last 14 nucleotides from the 3' end of 16S RNA (the vicinity of the "Shine Dalgarno" position) of *T. thermophilus*; bases 2646-2667, the " α-sarcin binding site" on 23S RNA of *T. thermophilus* and *H. marismortui* and bases 1422-1432, the "thiostrepton binding site", of the latter.

6. TOWARDS THE INTERPRETATION OF CRYSTALLOGRAPHIC MAPS

Assuming successful phasing and the availability of the sequences, significant difficulties in the interpretation of the electron density maps of the ribosomal particles are expected. Our ability to insert reactive sites (e.g., -SH) at desired locations on selected ribosomal components provides means for inserting powerful markers, as the localization of the heavy atom in the electron density map should not only facilitate phasing, but also provide us with flags and markers for chain tracing.

We expect to interpret the electron density map in an iterative fashion. We shall first focus on the determination of low resolution (20-30 Å) envelopes. At this stage it should be possible to localize the heavy atom clusters and since their binding sites on the ribosome are known, also to localize the components to which they are bound. The subsequent medium resolution (8-20 Å) electron density map is likely to reveal several internal features, such as regions rich in rRNA. For the interpretation of conformational elements of the rRNA we hope to benefit from the extensive non-crystallographic information about the relative locations of several double helical segments and loop-out regions.

Additional markers should be provided from comparisons of three-dimensional images reconstructed from unstained two-dimensional sheets by comparing images of native, mutated, depleted and chemically modified two-dimensional sheets (see above, 4.II). At this stage we may also be able to distinguish between less dense RNA regions and proteins. A large volume of data concerning the proximities of rRNA to specific ribosomal proteins is also available. In addition, we plan to take advantage of the available information concerning the relative positioning of centers of mass of the ribosomal proteins (obtained by neutron scattering combined with triangulation) as well as of the locations of surface ribosomal proteins (obtained by immuno-electron-microscopy).

I. Localization of Specific Sites and Functional Centers

It is conceivable that once the envelope of the ribosome has been elucidated, for the determination of the higher resolution structure, more conventional heavy atom derivatives, containing 4-6 heavy metal ions will be useful. These may also provide a tool for the identification of specific functional elements, such as following the path of the emerging nascent protein by attaching heavy atoms to its cysteins. Furthermore, the non-ribosomal components of the crystallized functional ribosomal complexes (tRNA and mRNA) may be used not only for improving crystal quality, but also as carriers of the heavy atom clusters. The crystallographic localization of these compounds should also shed light on their mode of interaction with the ribosome.

II. Sequencing the Ribosomal Proteins from *H. marismortui* and *T. thermophilus*

Protein chemical methods coupled with cloning techniques were used for sequencing the ribosomal proteins from *H. marismortui*. So far the primary structure of 52 ribosomal proteins have been established (Arndt et al., 1991; Scholzen and Arndt, 1991; Krömer and Arndt, 1991; Scholzen and Arndt, 1992). In these studies advantage has been taken of the conservation of the cluster arrangement of a large number of ribosomal genes.

Comparison of the primary structures of ribosomal proteins offers a promising tool for establishing phylogenetic relationships between the archaea, eucarya and eubacteria (Fig. 4). These comparisons may also provide valuable information about important functional sites, since regions crucial for assembly or for the translational process are expected to be conserved throughout evolution. Partial results show that the halophilic r-proteins are in general more similar to their eukaryotic than to the eubacterial counterparts, although some proteins are exclusively related to their eubacterial homologs. Interestingly, some of the halophilic r-proteins do not show similarities to any other proteins which have been sequenced so far.

Fifteen proteins of the 30S subunits from *T. thermophilus* have been partially sequenced and found to be of a high homology but different electrophoretic mobilities than those of their counterparts form *T. aquaticus, B. stearothermophilus* and *E. coli*. As the preferred nomenclature relates to sequence homologies, the original numbers assigned to some 30S r-proteins had to be reassigned (e.g. TthS14 is located on two-dimensional gels in the position of E. coli S21).

A low molecular weight protein containing 26 amino acids, of which 13 are basic and only one acidic, was detected in the 30S subunits. Interestingly, a homologous protein was detected in ribosomes from spinach chloroplast (Schmidt et al., 1992), but not in any eubacterial ribosome (Choli-Papadopoulou et al., 1992).

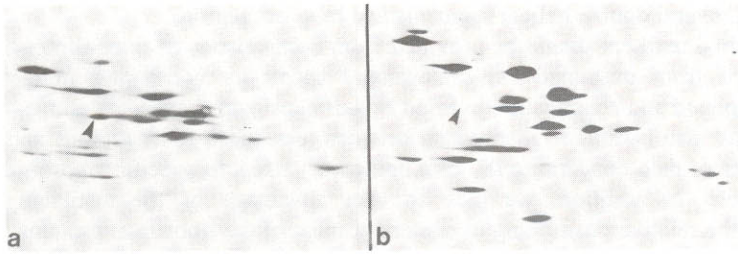

Figure 5. Two-dimensional gel electrophoresis of two samples of r-protein extracted from 50S subunits from *H. marismortui*. (a) Total proteins of the 50S subunits (the arrow points at protein HmaL1). (b) The fraction left after the separation of the HmaL1-23S RNA complex (the arrow points at the position of HmaL1).

III. The Isolation of in-situ Ribonucleoprotein Complexes

The knowledge of the accurate atomic structures of individual ribosomal components may be of instrumental assistance in the determination of the structure of ribosomes using the molecular replacement method. Therefore we purified a ribonucleoprotein complex with a defined composition, high stability and relatively low molecular weight, which may retain its native conformation.

After establishing a quantitative procedure for separating selected proteins from the ribosomal core, an internal complex composed of 23S RNA and protein L1 from *H.*

marismortui was isolated and characterized (Fig. 5). The rRNA fragments protected from RNase A digestion by protein HmaL1 show two regions of a high homology to the corresponding region of E. coli rRNA, despite the evolutionary distance. Stable heterologous complexes could be formed between the halophilic 23S RNA chain and protein L1 from E. coli, and between the rRNA of E. coli and the halophilic protein (Evers et al., submitted). Interestingly, although these complexes contain a non-halophilic component, they form under halophilic conditions. Protein L1 is one of the ribosomal proteins which act as translational repressors by binding to their own mRNA. In this capacity, there are considerable differences between the two bacteria, although their genes are clustered in a similar fashion. The production of the halophilic complex is currently being scaled up, aiming at its crystallization.

7. CONCLUDING REMARKS

The results reviewed in this chapter demonstrate that crystallographic studies on intact, modified, complexed and mutated ribosomal particles are well underway. From the early stages of this work, it has been clear that a straight-forward application of conventional concepts and techniques of macromolecular crystallography would not be adequate. Therefore we have devised an approach that combines the exploitation of the extensive information available on the genetic, functional and chemical properties of ribosomes for a rational design of innovative protocols in crystallization and in specific derivatization with multi heavy-atom clusters, together with the extension of the existing techniques of X-ray crystallography, image reconstruction, electron microscopy, neutron diffraction and cryogenics. We do expect that our efforts will lead to an understanding of the many functions of the ribosome at the molecular level.

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