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Crystallographic, Biochemical and Genetic Studies on Halophilic Ribosomes

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Summary

Ribosomal particles from *Haloarcula marismortui* were crystallized. The best crystals, diffracting to 2.9 Å resolution and yielding crystallographic data of reasonable quality were grown from the large ribosomal subunits. Attempts at crystallization of functional ribosomal complexes are in progress, benefiting from experience gained from crystals of ribosomes of an extreme thermophilic bacterium, *Thermus thermophilus*.

For obtaining phase information, a monofunctional reagent was prepared from an undecagold cluster, by attaching to it a chemically reactive handle, specific for sulfhydryl moieties. Heavy atom derivatives were prepared by binding this cluster to exposed sulfhydryls prior to the crystallization.

Cores of halophilic ribosomal particles, lacking four ribosomal proteins, were prepared using dioxane. All detached proteins could be fully reconstituted. However, blocking the -SH group of one of them (HmaL11), prevented its incorporation into the core particles. The so obtained depleted 50S subunits crystallize under the same conditions as native ones and show apparent isomorphism with them.

Most of the genes of several r-proteins were cloned. These are being used for sequencing as well as for providing new locations for binding heavy atom clusters by genetic insertion of cysteines on the ribosomal surface, according to accessibility data, obtained either chemically or by limited proteolysis.

A nucleoprotein complex of protein HmaL1 and a fragment of the 23S rRNA was isolated from ribosomes. Chimeric complexes were reconstituted with *E. coli* ribosomal components, indicating rather high homolgy, despite the evolution distance.

Key words: Haloarcula marismortui – Ribosomes crystallography – Undecagoldcluster – r-proteins: sequence of – Mutations of.

Introduction

The intricate and essential process of the enzymatic translation of the genetic information into proteins is performed by the universal cellular organelle, the ribosome. The ribosome is a nucleoprotein assembly, built of two subunits of unequal size, which associate upon initiation of protein synthesis. A typical bacterial ribosome (called also 70S, according to its sedimentation coefficient) contains about a quarter of a million atoms and is of a molecular weight of around 2.3 million daltons (1.45 and 0.85 for the large (50S) and the small (30S) subunits, respectively). Three chains of rRNA, of over 4500 nucleotides, account for about two-thirds of the ribosomes' mass, the rest are some 57–73 different proteins.

Eubacterial ribosomes have been the target of intensive biochemical, biophysical and genetic studies. These shed light on several functional and evolutionary aspects of

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Abbreviations: r-proteins stands for ribosomal proteins and rRNA for ribosomal RNA. E, B, T, and H in front of a name of a ribosomal protein shows its bacterial source (Estherichia coli; Bacillus stearothermophilus; Thermus Thermophilus and Haloarcula marismortui, respectively). L shows that the protein is of the large (50S) subunit, and S of the small (30S) one. The symbol: HmaL# indicates that this particular protein is homologous to protein # from E. coli.

protein biosynthesis and provided information on the spatial *in situ* proximity between several ribosomal components, the secondary structure of ribosomal RNA and on the approximate locations of some functional centers (reviewed in *Hill* et al., 1990; *Nierhaus* et al., 1993). However, for detailed understanding of the molecular mechanism of protein biosynthesis, a reliable molecular model is essential. In principle, such models may be obtained by X-ray crystallography.

The observations that under stressful conditions eukaryotic ribosomes pack orderly *in vivo,* and the hypothesis that these ordered forms provide the physiological mechanism for temporary storage of functionally active ribosomes, aimed at preserving their integrity and activity for an expected better future, stimulated crystallization of ribosomes from prokaryote. These were chosen because of their relative small size and because they can be produced in high purity and large quantities.

Numerous attempts to crystallize the traditionally studies ribosomes from *E. coli,* carried out in several laboratories, failed. However, intact, modified and complexed ribosomal particles from thermophilic or halophilic bacteria (reviewed in *Berkovitch-Yellin* et al., 1992, *Franceschi* et al., 1993) yielded crystals which diffract best to almost atomic resolution, 2.9 Å. These were proved suitable for X-ray (*von Böhlen* et al., 1991) and neutron (*Eisenstein* et al., 1991) crystallographic studies. All crystals of ribosomal particles grown in our laboratories, were obtained from biologically active particles and the crystalline material retained its integrity and activity for long periods, despite the natural tendency of the ribosomes to disintegrate.

Due to the weak diffracting power and the large unit cells of the ribosomal crystals, 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 ones. Under these conditions the crystals diffract with no observable decay for periods longer than needed for collecting a full set of data, and the irradiated crystals can be stored for months. To facilitate cryogenic data collection, special experimental procedures had to be designed. These accommodate the problematic features of the ribosomal crystals: fragility, sensitivity, thin edges, etc. (*Hope* et al., 1989; *Berkovitch-Yellin* et al., 1993).

The phasing of X-ray amplitudes remains the less predictable and most difficult part in structure determination. Phase information is crucial for constructing electron-density maps, and cannot be directly measured. The classical methods for phasing are MIR and SIR (Multiple and Single Isomorphous Replacement, respectively), both require specific and almost quantitative attachment of heavy atoms at a limited number of sites within the unit cell. Useful derivatives of proteins of average size consist of one or a few heavy-metal atoms. Much heavier compounds are needed for ribosomes. To reach the required electron-density we are using an undecagold cluster (GC, m. w. ~6200 Da), with a core of 11 gold atoms linked directly to each other. Conventional derivatization is a rather chancy process, which, for structures of complexity similar to that of ribosomes, may lead to multi-site binding. Therefore, for quantitative and site specific derivatization, a monofunctional reagent was developed. A chemically reactive handle of a limited flexibility and length (about 4.5 Å), imitating the peptide bond, was attached to the undecagold cluster. The end of this arm is a moiety, specific for binding to free sulfhydryls: maleimido or iodoacetyl. The first is the traditional reagent used for identifying sulfhydryls, the second was introduced to avoid cirality (*Weinstein* et al., 1989, *Franceschi* et al., 1993). Simulation studies showed that this cluster is of adequate phasing power at low and medium resolution, providing quantitative binding and reasonable isomorphism (*N. Volkmann* and *H. Bartels,* unpublished).

Preliminary derivatization experiments were performed on 50S subunits from *Bacillus stearothermophilus,* although they diffract to rather limited resolution (Table I). One ribosomal protein, BL 11, was removed by mutagenesis (*Schnier* et al., 1990) or by a stepwise addition of salts (*Gewitz* et al., 1987). The cluster was bound quantitatively to the isolated ribosomal protein, which was, in turn, reconstituted into mutated ribosomes, lacking it. The crystals of the so obtained fully derivatized particles yielded data of reasonable quality (*Weinstein* et al., 1989; *Yonath* et al., 1990; *Franceschi* et al., 1993), currently used in phasing attempts (*H. Bartels, H. A. S. Hansen, Z. Berkovitch-Yellin, I. Agmon, P. Rheses, W. S. Bennett, A. Zaytzev-Bashan, I. Levin, F. Schlünzen, N. Volkmann, A. Dribin* and *A. Yonath,* to be published).

In this chapter we describe our crystallographic studies on ribosomal particles from *Haloarcula marismortui.* We focus on our attempts at the derivatization of these crystals, and relate to the design of sophisticated procedures, specific for the halophilic ribosomes. We also highlight the interplay between the crystallogrphic, biochemical, functional and genetic studies and refer to the isolation of an internal nucleoprotein substructure.

Materials and Methods

Ribosomes were prepared according to (*Shevack* et al., 1985).

The crystals of the 50S subunits from *H. marismortui* were grown by vapor diffusion in Linbro dishes at 19 °C from 6–8 microliters of solution of 1.6 M KCl, 0.5 M NH_4Cl, 0.01 M $MgCl_2$, containing traces of non-physiological additives: 5–7% polyethylene glycol (m. w. 6000) and 1–2 mM $CdCl_2$. The crystals were exposed to X-rays when immersed in solutions of 3 M KCl, 0.5 M NH_4Cl and 0.01 M $MgCl_2$, with the same additives and 18% ethylene glycol, serving as a cryogen.

The procedures for crystallographic data collection and for the evaluation of these data are described in (*von Böhlen* et al., 1991; *Berkovitch-Yellin* et al., 1993).

The monofunctional undecagold cluster was prepared according to (*Weinstein* et al., 1989, 1992; *Franceschi* et al., 1993).

The detachment of selected ribosomal proteins from the 50S and 30S subunits of *H. marismortui* by dioxane is described in (*von Böhlen* et al., 1991). The detached proteins were incorporated into the depleted core particles by incubating both components at 55 °C for 40 minutes. A few proteins could also be de-

tached from the 50S subunits by lowering the salt concentrations to 0.5 M KCL, 0.0 M $MgCl_2$ and 0.10 Tris-HCl pH = 7.0.

The sequencing of the halophilic ribosomal proteins is described in (*Arndt* et al., 1991). For the overexpression of selected r-proteins, their genes were cloned from a λ-genomic-library or by the Polymerase Chain Reaction (*Mullis* et al., 1986). The genes were cloned in pET11d and overexpressed in *E. coli* BL21 (DE3 pLysS) (*Studier* et al., 1990). The overexpressed proteins were purified by ion-exchange chromatography (*Köpke* et al., 1990). The isolation and characterization of the internal ribonucleoprotein complex, containing HmaL1 and a fragment of 23S RNA, is described in (*Franceschi* et al., 1993). For the preparative scale production of this complex, HmaL1 was cloned, overexpressed and isolated via DEAE-Sepharose CL6B chromatography. The DNA coding for the fragment of rRNA protected by HmaL1 was obtained from chromosomal DNA using PCR. One of the oligo nucleotides used for this reaction contained sequence as that of the T7 RNA polymerase promoter. The fragment at the 5′ end, obtained by the PCR reaction, was subsequently transcribed *in vitro* with T7 RNA polymerase using the procedure of (*Milligan* and *Uhlenbeck*, 1989).

Exposed sulfhydryls were determined on both the large and small subunits, as described in (*Weinstein* et al., 1989; *Kruft* and *Wittmann-Liebold,* 1991). Preliminary attempts to label and identify exposed amino groups are currently being developed.

The *Haloarcula marismortui* mutant lacking protein HmaL11 was obtained by UV irradiation of cells grown in complete medium at 37 °C, until they had reach the middle logarithmic phase.

The cells were diluted to 1,2 A_{240nm}/ml, 5 ml of this cell suspension were placed in a petri dish (8.5 cm diameter) and irradiated for 15 min. The distance between the UV lamp (Sylvania G8T5) and the cell suspension was 24 cm. After the irradiation 0,15 A_{240} of the cell suspension was plated (under red light) and grown in the darkness for 17 days at 37 °C in full-medium plates containing two different concentrations of the antibiotic thiostrepton (0,5 μg/ml or 1,0 μg/ml).

Resistant clones (100 colonies) were analyzed via 2D-gel electrophoresis (*Strom* and *Visentin* 1973). One clone was found lacking protein HmaL11, these cells could resist up to 2,5 μg/ml of thiostrepton, but were not dependent on it for its growth. By partial reconstitution of the 50S subunits with split proteins (*von Böhlen* et al. 1991), HmaL11 could be incorporated into the mutant ribosomes. However, the mutated cells had the tendency to revert to the wild type with a frequency higher than expected.

Results and Discussion

1. The structural superiority of the halophilic and thermophilic ribosomes

It was found that the quality of the crystals is directly linked to the level of extremesness in the natural environment of the bacterium. Thus, ribosomes from normal eubacteria or from moderate halophiles did not crystallize, and those from extreme thermophiles crystallize better than those from moderate ones. We relate this property to the higher stability of the thermophilic and halophilic ribosomes, which are otherwise quite sensitive, flexible and unstable. It is likely that the harsh environmental conditions, imposed on the halophilic and thermophilic bacteria, resulted in acquiring a rather high stability, even in isolation.

a. The ribosomes from Eubacteria: B. stearothermophilus and T. thermophilus

The best characterized bacterial ribosomes are those of *E. coli.* For this reason, we, as well as others, have invested considerable effort in attempts to crystallize them. Unfortunately, only micro crystals could be grown from these ribosomes (*Wittmann* et al., 1982), probably due to their instability. In contrast to the poor results in the crystallization of ribosomal particles from *E. coli*, all three naturally occurring ribosomal particles (30S, 50S and 70S) from two thermophilic bacteria, *B. stearothermophilus* and *T. thermophilus* were crystallized (Table I, and in *Yonath* et al., 1990; *Volkmann* et al., 1989; Trakhanov et al., 1989; *Hansen* et al., 1990). One of these forms, of 50S ribosomal subunits from the moderate thermophile, *B. stearothermophilus,* diffract to rather low resolution (*Yonath* et al., 1984; *Müssig* et al., 1989). However, they are of interest due to the possibility of obtaining, by thiostrepton mutagenesis, a mutant lacking protein BL11 (*Schnier* et al., 1990). As mentioned in the introduction, these mutated particles were used for the preparation of specific heavy-atom derivatives. Furthermore, complexes of these subunits with tRNA and short nascent polypeptide chains crystallize under the same conditions as the native particle do (*Gewitz* et al., 1988).

So far, all crystals of 70S ribosomes (from *E. coli, B. stearothermophilus* and *T. thermophilus*) diffract to low resolution. Given the correlation between the functional activities of samples of 30S or 50S subunits and the quality of the crystals obtained from them, we believe that the poor internal order of the crystals of 70S particles is due to inherent conformational and/or functional heterogeneity of the 70S tight couples used for crystallization. Indeed, crystals of complexes mimicking a defined stage in protein biosynthesis, with a short mRNA (an oligomer of about 35 uridines) and approximately two $phetRNA^{phe}$ molecules, diffract significantly better than any other crystals of 70S particles that we, or others, have obtained so far (*Hansen* et al., 1990). Thus, useful diffraction data could be collected to 12 Å from crystals of this complex, compared to 22–24 Å obtained from the native 70S particles.

The suitability of the thermophilic ribosomes for structural studies was demonstrated not only in the crystallization of intact particles, but also in crystallographic studies on isolated ribosomal components. Previous difficulties encountered in attempts at crystallization of isolated components of *E. coli* ribosomes, coupled with the observations that some of the isolated components loose their *in-situ* conformation, led to the assumption that the conformations of all ribosomal components are dictated by their environment. The recent "quantum jump" in successful crystallization of isolated thermophilic r-proteins and fragments of r-RNA (reviewed in *Yonath* and *Franceschi,* 1993), suggests that some ribosomal components possess intrinsic characteristic fold which can be maintained even in isolation, providing they are not to be damaged during their preparation. Clearly, the chances of the thermophilic and the halophilic r-proteins to remain intact throughout the isolation procedure are higher than those of other sources.

Table 1. Characterized Three-Dimensional Crystals of Ribosomal Particles

Source	Grown from*	Cell Dimensions (Å)	Resolution (Å)
70S T.t.	MPD	$524 \times 524 \times 306$; $P4_12_12$	app. 20
70S T.t. # (+ mRNA & tRNA)	MPD	$524 \times 524 \times 306$; $P4_12_12$	12
30S T.t.	MPD	$407 \times 407 \times 170$; $P42_12_12$	7.3
50S H.m. ≤	PEG	$210 \times 300 \times 581$; C2221	2.9
50S T.t.	AS	$495 \times 495 \times 196$; $P4_12_12$	8.7
50S B.st. ◇	A	$360 \times 680 \times 920$; $P2_12_12$	app. 18
50S B.st. ◇≤	PEG	$300 \times 547 \times 377$; 111.9°; C2	9.5

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

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

\# A complex of 70S ribosomes, two molecules of $phetRNA^{phe}$ and an oligomer of 35 uridines (as mRNA).

◇ Same form and parameters for crystals of 50S from a mutant 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.

b. The ribosomes of the Archaebacteria Haloarcula marismortui

The Dead Sea, which has the highest salt concentration of any natural body of water in the world, supports the growth of several species of micro-organisms, all of which exhibit the unusual property of withstanding high salt concentrations. One of these is the archaebacterium *H. marismortui*. The ribosomes of this bacterium function under conditions which usually cause the dissociation of nucleoprotein assemblies and the denaturation of proteins. In addition, an apparent heterogeneity was detected in the genes encoding their rRNA (*Mylvaganam* and *Dennis*, 1993). Nevertheless, these particles yield the most exciting crystals.

i. Crystallization of the 50S subunits

A procedure has been developed for growing crystals in solutions of the lowest concentrations of salts essential to avoid the disintegration of the ribosomes, and collecting the crystallographic data under conditions similar to the physiological environment within the cells. An extensive search for the suitable ionic composition for the crystal growth solutions and the development of a sophisticated seeding procedure, led to the growth of six crystal forms of the 50S and one of the 30S subunits from this bacterium (*Shevack* et al., 1985; *Shoham* et al., 1986; *Makowski* et al., 1987; *von Böhlen* et al., 1991).

Crystals of the large ribosomal subunits may be obtained under conditions which are very close to the natural intracellular environment (*Shevack* et al., 1985; *Shoham* et al., 1986), but these are of very low quality. For improving them, advantage has been taken of studies on the physico-chemical properties of the halophilic ribosomes. These show that more than 3M salts are required for the activity of the halophilic ribosomes, but their integrity is maintained at significantly lower salt concentrations, providing a specific equilibrium between the mono and divalent ions is kept (*Shevack* et al., 1985) (Fig. 1).

The current crystals reach an average size of 0.3 × 0.3 × 0.08 mm, have cell dimensions of about 210 × 300 × 580 Å and $C222_1$ symmetry. The high resolution diffraction data sets contain a very large number of unique reflections. For instance, 1, 434, 786 reflections were measured for the resolution shell 3.5–50 Å, yielding 145, 249 unique

Fig. 1. Crystals of 50S ribosomal subunits from *Haloarcula marismortui*, grown within 6–12 days as described in Materials and Methods. Bar: 0.1 mm.

intensities. The evaluation of these data is still not a routine task, although some sophisticated computational tools have been developed specifically for this purpose. Nevertheless, 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 6 Å are in the range of 5–10% (*Berkovitch-Yellin* et al., 1993) and at 3 Å 12–15% (*A. Zaytzev-Bashan, H. Hansen, I. Agmon* and *Z. Berkovitch-Yellin*, to be published) (Fig. 2).

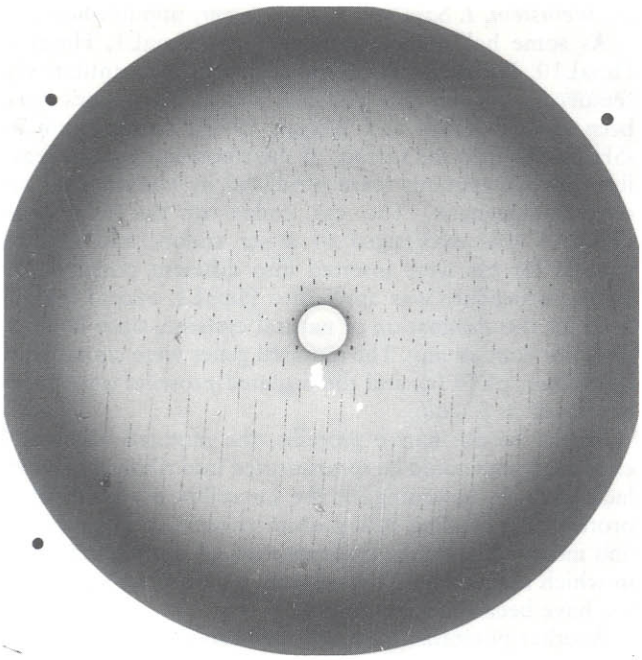

Fig. 2. 1° rotation photograph of a crystal similar to those shown in Fig. 1. The pattern was obtained at 90 K at Station F1/CHESS, operating at about 5.3 Gev and 50–80 Ma. The crystal to film distance was 220 mm, the diameter of the collimator = 0.1 mm; wave length = 0.9091 Å.

ii. crystals of other halophilic ribosomal particles and of functional complexes

Based on the observation that newly synthesized chains of polyphenylalanine can be attached to 50S subunits when dissociated from poly(U) programmed ribosomes of *H. marismortui*, we prepared a complex of these subunits with a short segment of poly(phe) (*Gewitz* et al., 1988). So far, only small crystals have been grown from this complex. These are currently being improved.

Crystals were also grown from the small (30S) ribosomal subunits of *H. marismortui*. These diffract rather well, but are either packed in very large unit cells, above 2000 Å (*Glotz* et al., 1987) or still somewhat disordered (*M. Laschever, I. Dunkel, S. Meyer, I. Sagi* und *G. Thoms*, unpublished). The latter are currently being improved.

2. Supporting Biochemistry and Genetics of Halophilic Ribosomes

a. In vitro association of the two ribosomal subunits

The most exciting particle, the 70S ribosome, yielded so far only micro crystals, most likely due to its marked tendency to dissociate to its two subunits. Preliminary observations point at a link between the stability of 70S particles and not only the ionic strength of their medium, but also on the type of the salts providing it. Advantage has been taken of studies showing that the ribosomal particles of a related halophilic bacterum, *Haloferax mediterranei*, require ammonium sulfate for their reconstitution (*Sanchez* et al., 1990). Thus, to aboid dissociation, the halophilic 70S ribosomes were isolated and kept in a buffer containing 1.9 M of this salt. Encouraged by the marked improvement in the internal order of crystals of complexes of 70S from *T. thermophilus*, mimicking a defined stage in protein biosynthesis (see above, Table I and in *Hansen* et al., 1990), we are attempting the design and crystallization of similar complexes of halophilic ribosomes. Therefore, the purification of several halophilic tRNA molecules has been initiated (together with *M. Safro* and *L. Reshetnikova*).

b. Quantitative removal of selected ribosomal proteins and reconstitution of active particles

Severe difficulties were encountered in attempts to adapt the procedure developed for the derivatization of the crystals of the 50S subunits form *B. stearothermophilus* (*Weinstein* et al., 1989, 1992) to the halophilic ribosomes. The significant resistance of the halophilic ribosomes to mutations (*Amils* et al., 1990) dictates almost exclusively the preparation of core particles by *in vitro* detachment of selected ribosomal proteins. One exception is a mutant of *H. marismortui*, lacking protein HmaL11, which was obtained by irradiation of the cells with ultra violet-light and posterior selection in medium containing the antibiotic thiostrepton (see materials and methods).

Using dioxane as a splitting agent, five ribosomal proteins were removed quantitatively by dioxane from the 50S subunits and two from the small one, all of which could be fully reconstituted with the core particles. One of these proteins, HmaL11, binds reagents specific to -SH groups, but the derivatized protein could not be incorporated into the core particles. In this way cores of 50S subunits, depleted of protein HmaL11, were obtained. The relative ease of the genetic and biochemical detachment of L11 from the halophilic as well as from various bacterial ribosomes poses the interesting question as to the role of this protein in the translational process. Clearly, it displays rather weak *in situ* interactions.

The ribosomal subunits lacking protein HmaL11 crystallize under the same conditions as the native 50S subunits and show apparent isomorphism with them, indicating that the removal of this protein caused neither major conformation changes in the ribosome, nor disturbances in the crystal's network. The crystals of the depleted par-

ticles reach average size of $0.2 \times 0.2 \times 0.05$ mm, and diffract well to 10 Å resolution. Thus, in later stages, these crystals should facilitate the interpretation of the electron density maps, by providing the location of HmaL11. This information is expected to be extracted from maps constructed from the differences between the diffraction of the native and depleted particle.

Decreasing the concentration of the salts cause a total detachment of the ribosomal proteins of several halophilic bacteria (*Sanchez* et al., 1990). Similar procedures were found to be only partially suitable for selective dissociation of ribosomal proteins from the large subunit (*S. Weinstein* and *M. Laschever,* unpublished) and not at all for the small one (*I. Sagi,* unpublished).

c. Sequencing the halophilic ribosomal proteins

Traditional methods coupled with cloning techniques are being employed for sequencing the ribosomal proteins from *H. marismortui.* So far the sequences of 45 ribosomal proteins have been established (*Arndt* and *Steffens,* 1992). In these studies advantage has been taken of the conservation of the cluster arrangement of a large number of ribosomal genes in eubacteria and archaebacteria (*Kromer* and *Arndt,* 1991).

Comparison of the primary structures of ribosomal proteins offers a promising tool for establishing phylogenetic relationships between the archaea, eucarya and eubacteria. 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 counterparts than to the eubacterial ones, although some proteins are exclusively related to their eubacterial homologues. Interestingly, some of the halophilic r-proteins show no similarities to any other proteins which have been sequenced so far (*Scholzen* and *Arndt,* 1992).

d. Cloning, over-expression and site directed mutagenesis of the halophilic ribosomal proteins

For further insertions of exposed cysteines on the surface of the ribosome we exploit the advanced stage of genetic sequencing. We also benefit from the efforts at mapping of the surface of the halobacterial ribosome, performed either by limited proteolysis (*Kruft* and *Wittmann-Liebold,* 1991), or by biochemical procedures for the determination of the number of the exposed amino groups (*S. Weinstein, I. Sagi* and *M. Laschever,* unpublished).

As some halophilic r-proteins (e.g. HmaL1, HmaL6, HmaL10, HmaL11 and HmaL12), can be quantitatively 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 site 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 need for the elimination of natural cysteine, since HmaL1 does not contain any. The mutated genes were cloned into the vector, pET11d, and the mutated proteins were over-expressed in *E. coli.*

Similar to the native proteins, the mutated and over-expressed ones could be reconstituted into ribosomal cores lacking them. Screening for 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 33 is a cysteine into core 50S particles, have been obtained (Fig. 3).

Another potential approach for derivatization is to exchange a selected ribosomal protein, which does not pos-

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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	-	-	-	-	+

Fig. 3. Genetic insertions of cysteines into protein HmaL11. Amino acid sequence of protein HmaL11.
Four different mutant proteins were produced, namely (1) HmaL11mut11, (2) HmaL11mut11/128, (3) HmaL11mut11/128/142 and (4) HmaL11mut11/33/128.

sess an exposed -SH group, with its homologue from another bacterial source, which contains such moiety. Using this approach, protein L12 from the ribosome of *Methanococcus vannielii* was incorporated into core particles obtained by depleting ribosomes of *H. marismortui* of the homologous one, HmaL12 (*Köpke* et al., 1990).

e. Internal nucleo-protein complexes

The knowledge of the accurate atomic structures of individual ribosomal components may be of instrumental assistance in the determination of the structure of the ribosome using the molecular replacement method. Therefore we purified a ribonucleoprotein complex with a defined composition, containing protein HmaL1 and the 23S rRNA fragment protected by it from RNase A digestion. This complex displays a high stability, hence it is expected to retain its native conformation (Fig. 4). The rRNA component of this complex shows two regions of a high homology to the corresponding stretch in *E. coli*, despite the significant evolutionary distance. Thus, it was found that stable heterologous complexes can be formed between the halophilic 23S RNA fragment and protein L1 from *E. coli*, and between the corresponding *E. coli* rRNA fragment and HmaL1 (*Franceschi* et al., 1993). It remains to be seen whether the preservation displayed by this complex indicates that the structural elements of the ribosome have been rather conserved throughout evolution.

Conclusions

Since we are investigating ribosomes from halophilic and thermophilic bacteria, we address not only the basic question of how the genetic information is translated into a protein chain, but also the broad topic of enzymatic activity at high salinity and elevated temperatures. We also relate to the interplay between harsh environmental conditions and the stability and integrity of multi-components cellular assemblies. We hope that our studies will lead to a better understanding of the forces holding together proteins and nucleic acids at high salt concentrations and shed some light on evolution.

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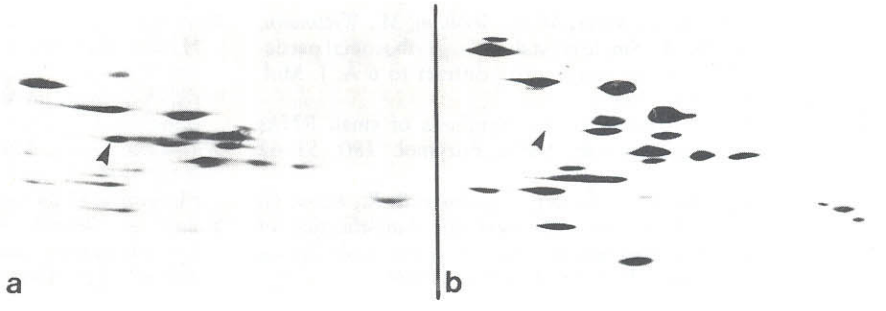

Fig. 4. Two-dimensional gel electrophoresis of two samples of r-protein extracted from 50S subunits from *H. marismortui*. (a) Total proteins of the 50S subunits (the arrow points at protein HmaL1). (b) The fraction left after the separation of the HmaL1-23S RNA complex (the arrow points at the position of HmaL1).

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