Characterization and crystallization of ribosomal particles from *Halobacterium marismortui*

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Ribosomes and their subunits have been isolated from *Halobacterium marismortui*, an extremely halophilic bacterium from the Dead Sea. The stability and functional activity of the subunits were tested under a wide range of salt conditions. Three-dimensional microcrystals of the large ribosomal subunits have been obtained. Electron microscopy of positively stained thin sections of these crystals showed that the particles are closely packed with approximate cell constants of 310 × 350 Å.

Halobacteria *Ribosome* *Large subunits* *3-dimensional crystal* *Ribosomal particle stability* *Poly(U) system activity* *Unit cell dimension*

1. INTRODUCTION

We have initiated X-ray diffraction and 3-dimensional image reconstruction studies on crystals of ribosomal particles and have so far obtained several 3-dimensional crystal forms and 2-dimensional sheets of ribosomal particles from *Bacillus stearothermophilus* or *Escherichia coli* [1–5].

Since these crystals were obtained from organic solvents, which introduce some technical difficulties in the handling of the crystals, we have also directed our efforts towards the crystallization of ribosomes which are stable under high salt conditions and might therefore be crystallized from ammonium sulfate or other salts.

It is known that ribosomal particles from halophilic bacteria are stable in high concentrations of salts [6–9], in contrast to other ribosomes, from which many proteins dissociate under these conditions. Most ribosomal proteins from halobacteria are acidic [7–9] whereas those of other organisms are very rich in basic amino acids [10]. This raises interesting questions about the nature of the protein-RNA interactions in these particles.

Here, we report studies on the isolation, stability and functional activity of ribosomal particles from extremely halophilic bacteria in a wide range of salt conditions. We further succeeded in the crystallization of the large ribosomal subunits of *Halobacterium marismortui* isolated from the Dead Sea.

2. MATERIALS AND METHODS

2.1. *Large scale growth of cells*

The original inoculum of *H. marismortui* [11] was a gift from Drs M. and B.Z. Ginsburg. The bacteria were grown according to Mevarech et al. [12] using a 100 l or 300 l fermenter in the following medium: 208 g NaCl, 46 g anhydrous $MgSO_4$ 0.125 g $MnCl_2$ and 10 g Difco yeast extract per l. After sterilization, 0.5 g $CaCl_2$ per l was added and the pH was adjusted to 7.2 with 0.2 M NaOH. Cells were harvested in a continuous flow centrifuge in the log phase after approx. 45 h at 37°C. The yield was about 3 g cells (wet wt) per l. The harvested cells were stored in portions of 100 g at −80°C.

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2.2. *Isolation of ribosomes and their subunits*

The isolation of ribosomes followed essentially the procedure described by Visentin et al. [8] with several modifications. 100 g of cells were disrupted by grinding (4 × 2 min) with 200 g aluminium powder (Alcoa) at 4°C in a precooled mortar. The paste was extracted with a buffer [13] containing 3.4 M KCl, 100 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6), and 6 mM 2-mercaptoethanol. The cell debris was removed by an initial centrifugation at 27000 × g for 15 min, followed by another one at 65000 × g for 40 min. Ribosomes were pelleted at 255000 × g for 4 h in a Spinco 60 Ti rotor. Tubes were inverted on paper for 10–20 min to allow the reddish, gelatinous material covering the ribosomal pellet to slough off, and the surface of the pellet was rinsed with the buffer. After resuspension and low-speed centrifugation, the ribosomes were rapidly frozen in liquid nitrogen and stored in small portions at −80°C.

For the isolation of subunits the ribosomes were first dialyzed against a dissociation buffer (2.7 M KCl, 0.45 M NH_4Cl, 1 mM $MgCl_2$, 0.02 M Tris-HCl (pH 8.0), 6 mM 2-mercaptoethanol) and then loaded onto a gradient containing 6–35% sucrose (w/v) in the dissociation buffer. After centrifugation in a Ti15 zonal rotor for 16 h at 25000 rpm and 9°C the fractions with the 30S or 50S subunits were pooled, and the Mg concentration was increased to 10 mM. The sucrose was removed in one of two ways: (a) After dialysis against the dissociation buffer which contained 10 mM (instead of 1 mM $MgCl_2$), the subunits were concentrated in an Amicon Diaflo ultrafilter with a PM-10 membrane. (b) The pooled fractions with the subunits in a volume of up to 1 l were passed through a Sephacryl 200 (superfine) column (75 × 8 cm) equilibrated with the dissociation buffer containing 10 mM $MgCl_2$. Sucrose-free subunits were eluted after 2–3 h with a small increase of the volume. The subunits were concentrated by ultrafiltration as described and divided into small portions which were quickly frozen in liquid nitrogen and stored at −80°C.

2.3. *Poly(U) assay and fraction S50*

The determination of the biological activity of the ribosomal particles in a poly(U) in vitro system was carried out as described by Saruyama and Nierhaus [14].

For the preparation of the fraction S50, 100 g of cells were ground with 200 g of aluminium powder (Alcoa) for 30 min in a Retsch mill at 4°C without addition of buffer. The paste was homogenized with 200 ml of a buffer containing 3.4 M KCl, 100 mM Mg-acetate, 10 mM Tris-HCl (pH 8.0), and 6 mM 2-mercaptoethanol for 10 min in the same mill. The homogenate was centrifuged at 27000 × g for 15 min and the supernatant at 50000 × g for 20 h at 4°C. The upper two-thirds of the supernatant (S50) were removed and divided into several aliquots which were frozen in liquid nitrogen and stored at −80°C.

2.4. *Crystallization*

Microcrystals were obtained in hanging drops with the vapour diffusion technique in Linbro tissue culture trays under the following conditions: 50 S subunits frozen in a buffer containing 3.0 M KCl, 0.5 M NH_4Cl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 6 mM 2-mercaptoethanol were thawed and diluted with the same buffer to a concentration of 100 A_{260}. Solid polyethylene glycol 6000 (Merck) was added to a final concentration of 5%. This was followed by addition of $MgCl_2$ to several aliquots covering the range of 100–250 mM Mg^{2+}. Drops of 9 μl were spotted onto plastic coverslips, inverted and placed on top of a well in a Linbro tray. Each well contained 1 ml of 9% polyethylene glycol in the buffer described above. Microcrystals appeared after storage of the trays at 4°C for 4–10 days.

2.5. *Electron microscopy and optical diffraction*

The structure of the microcrystals was examined by fixing the contents of the hanging drops in 0.7% glutaraldehyde followed by dehydration in an acetone series and embedding in resin ERL 4206 [1,15]. Thin sections of the embedded material were cut in a microtome, and mounted on a copper grid which was covered by a Formvar film and carbon coated. The mounted sections were stained first with 4% tungstic acid and then with 5% uranyl acetate. Electron micrographs were taken on a Phillips EM 400 at 17000-, 22000- and 28000-fold magnifications. Diffraction patterns of the electron micrographs were obtained with an optical diffractometer.

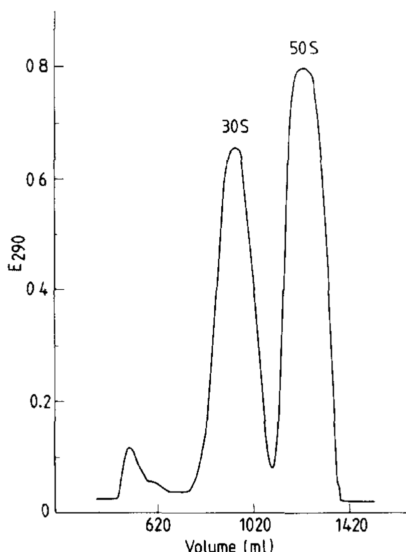

Fig.1. Separation of the ribosomal subunits of *H. marismortui* by zonal centrifugation under the conditions described in section 2.

3. RESULTS AND DISCUSSION

3.1. *Isolation and stability of ribosomal subunits*

The separation of 30 S and 50 S ribosomal subunits of *H. marismortui* by zonal centrifugation under the conditions described in section 2 is excellent (fig.1) and better than that of ribosomal subunits from any other species we have studied so far.

To test the stability of the 30 S ribosomal subunits they were dialyzed overnight at 4°C against buffers containing various salt concentrations as described in table 1. After the dialysis, two aliquots were removed: one was tested for biological activity in the poly(U) system, and the other was subjected to high speed centrifugation. The pelleted particles were resuspended and then tested for poly(U) activity. In this way it was checked whether proteins which are possibly removed from the ribosomal particles are readsorbed under the conditions of the poly(U) assay. Since this was not the case, the results from the poly(U) activity tests with and without centrifugation were averaged.

As seen in table 1, the concentration of KCl can be decreased from 3 to 0.1 M without loss of poly(U) activity; i.e., the 30 S subunits remain intact under these conditions. Even a further decrease of the KCl concentration has only a small effect on the activity. On the other hand, the concentration of NH_4Cl cannot be decreased below

Table 1

Activity of 30 S subunits in the poly(U) system after dialysis against various salts and addition of 50 S subunits

Salt in buffer A[a]	Activity (%)	Salt in buffer B[b]	Activity (%)	Salt in buffer C[c]	Activity (%)	Salt in buffer D[d]	Activity (%)
3.0 M KCl	100[e]	0.5 M NH_4Cl	100	50 mM $MgCl_2$	100	2 M $(NH_4)_2SO_4$	100
2.5 M KCl	100	0.25 M NH_4Cl	73	15 mM $MgCl_2$	75	1 M $(NH_4)_2SO_4$	86
2.0 M KCl	100	0.12 M NH_4Cl	68	6 mM $MgCl_2$	35	0.5 M $(NH_4)_2SO_4$	83
1.5 M KCl	100	0.05 M NH_4Cl	40	1 mM $MgCl_2$	3		
1.0 M KCl	100			0.3 mM $MgCl_2$	0		
0.5 M KCl	100						
0.2 M KCl	100						
0.1 M KCl	100						
0.05 M KCl	93						
0 M KCl	90						

[a] Buffer A: 20 mM Tris (pH 8.0), 50 mM $MgCl_2$, 0.5 M NH_4Cl, 6 mM β-mercaptoethanol
[b] Buffer B: 20 mM Tris (pH 8.0), 50 mM $MgCl_2$, 50 mM KCl, 6 mM β-mercaptoethanol
[c] Buffer C: 20 mM Tris (pH 8.0), 50 mM KCl, 0.5 M NH_4Cl, 6 mM β-mercaptoethanol
[d] Buffer D: 10 mM Tris (pH 8.0), 30 mM $MgCl_2$, 100 mM KCl, 6 mM β-mercaptoethanol
[e] 100% corresponds to 19700 cpm in the experiment with KCl variation, to 20900 cpm with NH_4Cl, to 18100 cpm with $MgCl_2$, and to 17300 cpm with $(NH_4)_2SO_4$. All poly(U) tests were done under the same conditions, as described in section 2

0.5 M and that of $MgCl_2$ not below 50 mM without impairing the poly(U) activity.

To assess the feasibility of using lower salt concentrations in future crystallization experiments, an attempt was made to exchange NH_4Cl by $(NH_4)_2SO_4$ without impairing the poly(U) activity. This proved possible, since even in the complete absence of NH_4Cl, but in the presence of 2 M $(NH_4)_2SO_4$, the 30 S subunits remain functionally active (table 1).

Similar experiments were also performed to test the stability of the 50 S subunits. A decrease of the KCl concentration from 3.0 to 2.5 M KCl during the dialysis overnight caused a 50% loss of the activity in the poly(U) assay. A further decrease from 2.5 to 0.2 M KCl did not have any effect; i.e., the activity remained at approx. 50%. A variation of the $(NH_4)_2SO_4$ concentrations between 0.5 and 3.0 M did not impair the activity of the 50 S subunits. These results show that even a large variation in the concentration of ammonium sulfate leaves the 50 S subunits intact and functionally active.

3.2. *Crystallization and electron microscopy*

The crystallization experiments described in section 2 resulted in needlelike crystals of 50 S ribosomal subunits. An electron micrograph of a positively stained thin section through a crystal is shown in fig.2. Unit cell dimensions of approx. 310 × 350 Å were determined. These are similar to the cell constants we have obtained for crystal form number 3 of *B. stearothermophilus* 50 S subunits [3].

Experiments are in progress to improve the size and quality of the crystals by various approaches, e.g., by seeding techniques [16].

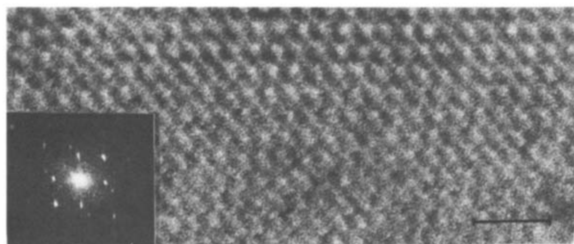

Fig.2. Electron micrograph of a positively stained section through an embedded crystal and the diffraction pattern. Bar length = 1000 Å.

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REFERENCES

[1] Yonath, A., Müssig, J., Tesche, B., Lorenz, S., Erdmann, V.A. and Wittmann, H.G. (1980) Biochem. Internat. 1, 428–435.
[2] Wittmann, H.G., Müssig, J., Piefke, J., Gewitz, H.S., Rheinberger, H.J. and Yonath, A. (1982) FEBS Lett. 146, 217–220.
[3] Yonath, A., Tesche, B., Lorenz, S., Müssig, J., Erdmann, V.A. and Wittmann, H.G. (1983) FEBS Lett. 154, 15–20.
[4] Arad, T., Leonard, K., Wittmann, H.G. and Yonath, A. (1984) EMBO J. 3, 127–131.
[5] Yonath, A., Bartunik, H.D., Bartels, K.S. and Wittmann, H.G. (1984) J. Mol. Biol. 177, 201–206.
[6] Bayley, S.T. and Kushner, D.J. (1964) J. Mol. Biol. 9, 654–669.
[7] Bayley, S.T. (1966) J. Mol. Biol. 15, 420–427.
[8] Visentin, L.P., Chow, C., Matheson, A.T., Yaguchi, M. and Rollin, F. (1972) Biochem. J. 130, 103–110.
[9] Matheson, A.T., Yaguchi, M., Nazar, R.N., Visentin, L.P. and Willick, G.E. (1978) in: Energetics and Structure of Halophilic Organisms (Caplan, S.R. and Ginsburg, M. eds) pp.481–501, Elsevier/North-Holland Biomed. Press, Amsterdam.
[10] Giri, L., Hill, W.E., Wittmann, H.G. and Wittmann-Liebold, B. (1984) Adv. Prot. Chem. 36, 1–78.
[11] Ginzburg, M., Sachs, L. and Ginzburg, B.Z. (1970) J. Gen. Physiol. 55, 187–207.
[12] Mevarech, M., Eisenberg, H. and Neumann, E. (1977) Biochemistry 16, 3781–3785.
[13] Bayley, S.T. and Griffiths, E. (1968) Biochemistry 7, 2249–2256.
[14] Saruyama, H. and Nierhaus, K.H. (1985) submitted.
[15] Spurr, A.R. (1969) J. Ultrastruct. Res. 26, 31–43.
[16] Thaller, C., Weaver, L.M., Eichele, G., Wilson, E., Karlsson, R. and Jonasonius, J.N. (1981) J. Mol. Biol. 147, 465–469.