

Characterization and Preliminary Attempts for Derivatization of Crystals of Large Ribosomal Subunits from *Haloarcula marismortui* Diffracting to 3 Å Resolution

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An improved form of crystals of large (50 S) ribosomal subunits from *Haloarcula marismortui*, formally named *Halobacterium marismortui*, diffracting to 3 Å resolution, has been obtained by the addition of 1 mM-Cd²⁺ to the crystallization medium, which contained more than 1.9 M of other salts. The improved crystals, grown from functionally active particles to an average size of 0.3 mm × 0.3 mm × 0.08 mm, are isomorphous with the previously reported ones, which diffracted to 4.5 Å. They are of space group C222₁, cell dimensions $a = 210$ Å, $b = 300$ Å, $c = 581$ Å, and contain one particle in the asymmetric unit. Their superior internal order is reflected not only in their high resolution, but also in their reasonable mosaicity (less than 0.3°). In contrast to the previously grown crystals, the new ones are of adequate mechanical strength and survive well the shock-cooling treatment. Due to their weak diffracting power, all crystallographic studies have been performed with synchrotron radiation. At cryotemperature, these crystals showed no measurable decay for a few days of irradiation and a complete diffraction data set could be collected from a single crystal. Efforts for initial phasing by specific and quantitative derivatization with superdense heavy-atom clusters are in progress.

Keywords: ribosomes; *Haloarcula marismortui*; cryo-biocystallography; heavy-atom clusters

Aiming at the determination of the three-dimensional structure of the ribosome at the molecular level, we have crystallized ribosomal particles from several prokaryotes. Those that were found to be suitable for crystallographic analysis were grown from extreme halophilic or thermophilic bacteria, probably because of their relative stability (Yonath & Wittmann, 1989a; Hansen *et al.*, 1990; Berkovitch-Yellin *et al.*, 1991). The crystals that diffract to the highest Bragg resolution are of the large (50 S) ribosomal subunits from *Haloarcula marismortui* formally named *Halobacterium marismortui*. Several years ago we reported (Shevack *et al.*, 1985; Shoham *et al.*, 1986) diffraction to 13 to 18 Å (1 Å = 0.1 nm) from the first generation of a crystal form, which was subsequently improved,

first to a resolution limit of 6 Å (Makowski *et al.*, 1987), and later to 4.5 Å (Yonath & Wittmann, 1989a).

At ambient temperatures the X-ray ionizing radiation causes severe and rapid damage to these crystals, so that all reflections beyond about 18 Å resolution decay within a few minutes, a period shorter than the time required to obtain a single X-ray rotation or still photograph. In our initial crystallographic experiments (Makowski *et al.*, 1987), crystals were mounted in capillaries in arbitrary orientations, since they were too fragile to be aligned. None of the 263 crystals that were irradiated in an attempt to collect a complete data set were oriented so that their orthorhombic symmetry, C222₁, could be detected. Therefore, we were misled

into assigning parameters of the corresponding primitive unit cell, $P2_1$ (Makowski *et al.*, 1987).

We were able to overcome the problem of radiation sensitivity by collecting data at cryotemperature. Crystals were immersed in solutions containing all the components present in their original stabilizing solution, but modified by stepwise addition of a hydrophilic cryosolvent (ethylene glycol, to a maximum concentration of 18% (v/v) over 15 to 30 min). A single crystal, together with small amounts of its soaking solution, was placed between two glass films of a double-layer mini-spatulum, constructed to allow the orientation at which the long cell axis is parallel to the spindle direction (Yonath *et al.*, 1988; Hope *et al.*, 1989). The spatulum holding the crystal was shock-cooled by instant plunging into propane, at 90 to 100 K, and transferred at that temperature to the X-ray camera, where it was surrounded by a stream of nitrogen gas kept at similar temperatures throughout the entire period of data collection. At this temperature, the crystals hardly decay even when irradiated for days or stored for months, permitting the collection of a complete set of diffraction data from an individual crystal.

Despite the reasonable internal order of the previous crystals and the introduction of cryotemperature, we encountered considerable difficulties in collecting and processing their crystallographic data. Being multi-layered thin plates held together by rather weak forces, they were susceptible to the mechanical stresses of the shock cooling, and frequently broke during the preparation. Their high mosaicity (1 to 3°) dictated correspondingly large rotation ranges, which resulted in high background levels. This, coupled with a sharp fall-off in the

intensities at higher resolution and a severe fragmentation of the crystals, led to marked differences between the potential resolution, 4.5 Å, and the usable one, 7 to 9 Å.

To improve further the quality of the crystals, we systematically added to the crystallization media minute quantities of metal ions with a high coordination and/or organic materials. These efforts resulted in the growth of three new crystal forms (Fig. 1). The most effective additives were Cd^{2+} and β -octyl-glucoside. These additives had a significant influence on the internal order of the crystals accompanied by gross morphological changes, in accord with that observed for isolated proteins (McPhearson *et al.*, 1986) and small organic molecules (Berkovitch-Yellin *et al.*, 1985). These three new forms are of rather attractive morphology (Fig. 1), but only one of them, the subject here, displayed a significant improvement. The other two showed a lower internal order (both diffracted to 15 to 20 Å). It is noteworthy that the addition of tiny amounts of these additives were found to be essential for obtaining crystals from other ribosomal particles: 30 S and 70 S from *Bacillus stearothermophilus* and 70 S from *Thermus thermophilus* (Yonath *et al.*, 1990; Berkovitch-Yellin *et al.*, 1991).

We were guided by the complementary effects of the concentrations of monovalent and divalent ions on the growth of halobacteria, as well as by the role played by the Mg^{2+} concentration in crystallization of other ribosomal particles (Arad *et al.*, 1984; Makowski *et al.*, 1987). The influence of Mg^{2+} on the quality of the crystals may be attributed partially to its contribution to the integrity of the ribosomal particles. However, even optimal Mg^{2+} concentrations could not lead to the effect obtained by the

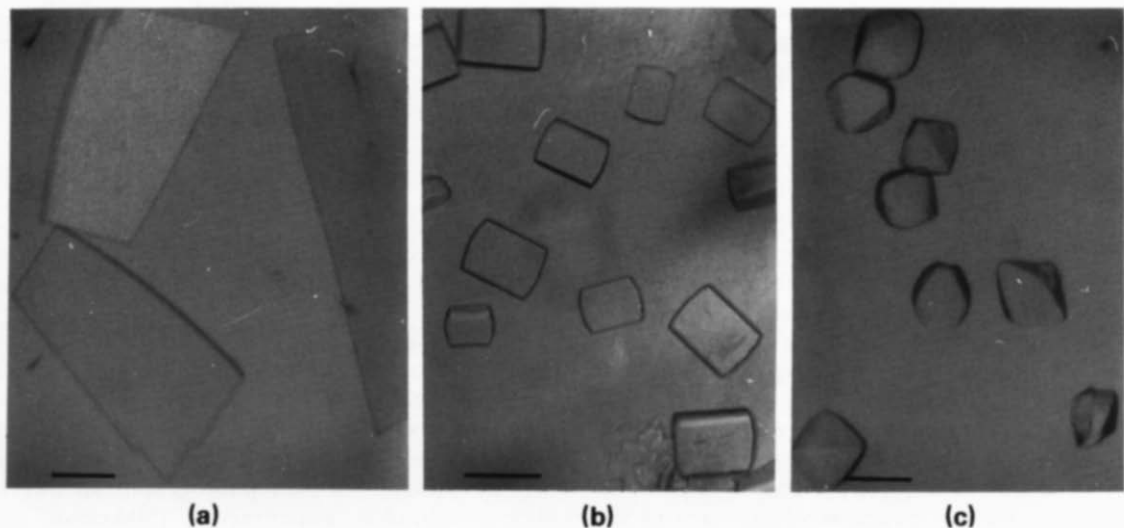


Figure 1. Crystals of 50 S ribosomal subunits from *H. marismortui* grown within 6 to 12 days by vapor diffusion in Linbro dishes at 19°C from 6 to 8 μl of the "basic solution", containing: 5 mg 50 S subunits/ml, 1.2 M-KCl, 0.005 M-magnesium chloride, 5 to 6% polyethylene glycol 6000 (pH 5.6) (0.1 M-acetate buffer). The following additives were added to the basic solution: (a) 0.001 M-cadmium chloride; (b) 0.01 mM- β -octyl-glucoside; (c) 0.00185 mM- CdCl_2 and 0.0074 mM- β -octyl-glucoside. All drops were equilibrated with 1 ml of the reservoir solution of 1.7 M-KCl and with all the other components of the drop. Bars represent 0.1 mm.

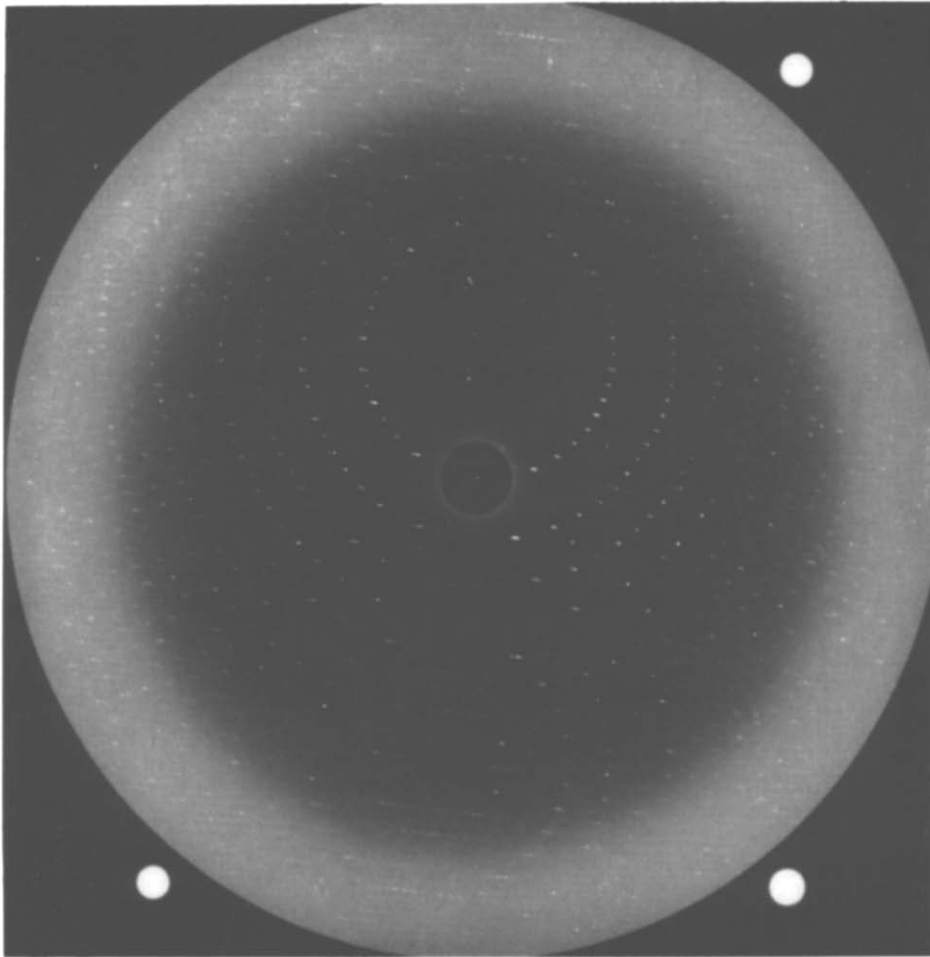


Figure 2. A 1° rotation photograph of a crystal similar to those shown in Fig. 1. The pattern was obtained at 90 K at Station F1/CHES, operating at about 5.3 GeV and 50 to 80 mA. The crystal to film distance was 220 mm, the diameter of the collimator was 0.1 mm and the wavelength was 0.9091 Å.

addition of 1 mM- Cd^{2+} to the otherwise most effective crystallization solution: the growth of crystals diffracting to 3 Å resolution (Fig. 2), with a reasonable mosaicity (0.2 to 0.3°) and a superb mechanical stability. We did not encounter cracked or fragmented crystals, as was the case for most of the previous ones, and we observed that the improved crystals survived the shock-cooling treatment well.

Like the crystals of other ribosomal particles grown in our laboratory, these crystals were obtained from biologically active ribosomal particles, by sophisticated individual seeding (Yonath & Wittmann, 1989b), and the crystalline material retained its integrity and biological activity for long periods.

For the derivatization of several ribosomal particles we have initiated the use of an undecagold cluster, consisting of a compact core of 11 gold atoms directly linked to one another (Jahn, 1989). Prepared as a monofunctional reagent, specific for sulfhydryl groups (Weinstein *et al.*, 1989) this cluster was covalently bound to several ribosomal particles prior to their crystallization. We optimized the yield and the specificity of cluster binding by determining the conditions for minimizing the

number of exposed $-\text{SH}$ moieties on the surface of the ribosomal particles. In this way we were able to fully derivatize the small subunits of *T. thermophilus* (Yonath *et al.*, 1990) without impairing their integrity and ability to crystallize. We were also able to obtain specific, however, not quantitative, binding of the gold cluster to either one or two sites on the large subunit from *H. marismortui*.

By its nature, direct binding of the cluster to intact ribosomal particles cannot be fully controlled. Therefore we have developed a procedure that was used for the derivatization of 50 S subunits of *B. stearothermophilus*. The cluster was bound to the cysteine residue of isolated ribosomal protein BL11, and the modified protein was, in turn, reconstituted into mutated core particles lacking this protein (Yonath *et al.*, 1987; Weinstein *et al.*, 1989).

Attempts to apply a similar approach to halophilic ribosomes are underway. For eubacterial ribosomes, quantitative removal of selected ribosomal proteins is achieved by mutagenesis (Dabbs, 1987) or by a stepwise addition of salts to ribosomal suspensions (Atsmon *et al.*, 1969; Gewitz *et al.*, 1987). The significant resistance of the halophilic

ribosomes to mutation dictates the preparation of core particles almost exclusively by *in vitro* detachment of selected ribosomal proteins. The natural salinity required to maintain the integrity and activity of halophilic ribosomes is so high that a substantial addition of salts is not possible. A total removal of the ribosomal proteins of several halophilic bacteria was achieved by decreasing the concentration of the salts (Sanchez *et al.*, 1990), but this procedure was found to be not usable for the selective dissociation of ribosomal proteins.

Dioxane was found to be a suitable agent for partial removal of several proteins from ribosomes of *H. marismortui*, all of which could be reconstituted quantitatively into the core particle. One of the removed proteins, HL11, binds reagents specific to -SH groups, but the derived protein could not be incorporated into the core particle. However, since in this way it is possible to remove quantitatively this protein from 50 S subunits under mild conditions, the resulting particle may be considered as a "minus" ribosome, which may be useful for preliminary phase determination. The halophilic 50 S core particles, depleted of HL11, crystallize under the same conditions as native 50 S subunits. Therefore, it seems that the removal of this protein does not cause conformational changes of the 50 S subunit.

On the basis of the observation that newly synthesized chains of polyphenylalanine can be attached to 50 S subunits when dissociated from poly(U) programmed ribosomes of *H. marismortui*, we have prepared a complex of these subunits with a short segment of this polypeptide (Gewitz *et al.*, 1988). This complex could be used for illuminating the exit path of the nascent protein, as well as for derivatization. So far only small crystals have been grown from this complex. These are currently being improved.

We have obtained crystals of the small (30 S) ribosomal subunits of *H. marismortui* that diffract rather well but are packed in very large unit cells, above 2000 Å (Glotz *et al.*, 1987). Crystallization efforts, aimed at obtaining more suitable crystals from these particles are underway. The most exciting particles, the 70 S ribosomes from *H. marismortui* have not been crystallized, due to its tendency to dissociate. Conditions for increasing the stability of the associated particles are currently being refined. Preliminary observations point at a possible link between the stability of 70 S particles and the total ionic strength of their medium, as well as the relative concentrations of mono- and divalent ions. Encouraged by the marked improvement in the internal order of crystals of complexes of 70 S (from *T. thermophilus*), mimicking a defined stage in protein biosynthesis (Hansen *et al.*, 1990), we are attempting the production and crystallization of similar complexes of halophilic ribosomes. A series of crystals (of halophilic 50 S, 30 S and 70 S particles) is expected to enable the investigation of the conformational changes that take place upon association of the ribosomal subunits, as well as

upon binding of components participating in protein biosynthesis.

On the other hand, elucidating the structures of 50 S ribosomal subunits from *H. marismortui*, *B. stearothermophilus* (Müssig *et al.*, 1989) and *T. thermophilus* (Volkman *et al.*, 1990), should facilitate a thorough comparison of this particle from radically different sources: moderate and extreme thermophiles, both belonging to eubacteria, along with extreme halophiles that are classified as archaeobacteria. Thus, we expect to shed light not only on the process of protein biosynthesis but also on fundamental aspects in evolution.

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