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THE EFFECT OF CRYOGENIC TREATMENT ON THE CELL DIMENSIONS OF RIBOSOMAL CRYSTALS

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SUMMARY

We report here our attempts at quantitative assessment of the effect of the shock freezing process on two properties of ribosomal crystals: shrinkage of the cell dimensions and isomorphism. We also refer to qualitative observations concerning the resolution and the mosaic spread of shock-frozen crystals.

Ever since we introduced cryo-temperature crystallographic data collection, we suspected that the inherent flexibility of the ribosome may not only allow significant shrinkage of the unit cell but also may lead to variability in its magnitude. However, independent determination of the cell dimensions of each part of a segmented crystal show that the shock cooling process was rather reproducible and did not harm the isomorphism.

These findings are most important for the continuation of our studies as they decrease our previous concern about the lack of isomorphism which may be caused by the mechanical and chemical stresses involved in the process of shock freezing.

I. Introduction

Ribosomes are the universal supramolecular assemblies responsible for the translation of genetic information, encoded in mRNA, into proteins. A typical bacterial ribosome contains more than 250.000 atoms, has a molecular weight of about 2.3×10^6 and a sedimentation coefficient of 70S. It is composed of two subunits of unequal size (small = 30S, m.w.:0.85×10^6, and large = 50S, m.w. = 1.45×10^6), which associate upon the initiation of the biosynthetic process. Bacterial ribosomes contain three chains of rRNA (about 5500 nucleotides), accounting for two thirds of its mass and 57-73 different proteins, depending on the species.

Systematic exploration of crystallization conditions, combined with sophisticated seeding, led to reproducible growth of ordered three-dimensional crystals of ribosomes, their intact subunits, their mutants and their functionally active complexes, diffracting best to 2.9 Å (von Böhlen et al., 1991). All crystals suitable for crystallographic studies are of ribosomal particles from halophilic or thermophilic bacteria, and in all cases, the crystalline ribosomal particles retain their integrity and biological activity for long periods despite their natural tendency to disintegrate rapidly. The large unit cell dimensions and the extremely weak diffracting power of the ribosomal crystals dictate the

use of intense synchrotron radiation for all steps in data collection, including the characterization of the crystals and the search for suitable derivatives (for review see Berkovitch-Yellin et al., 1992).

At ambient temperatures, the radiation damage of ribosomal crystals is so severe that most of the reflections beyond Bragg spacings of 10-15 Å decay almost instantaneously. Initially, before this property was noticed, this extreme sensitivity led to the incorrect conclusion that the measurable diffraction of all the ribosomal crystals was limited to that resolution. Only after observing the first indications for a higher resolution, a special procedure was designed, according to which precise alignment was skipped and each individual crystal was exposed only once. The combination of randomly oriented crystals, a high mosaic spread and single exposures resulted in wrong assignments of the space group, and, more seriously, in repeated failures to collect complete data sets, even when 263 were exposed (experiment performed at X11/EMBL/DESY, October, 1985).

The radiation damage of the ribosomal crystals was virtually eliminated by collecting the crystallographic data at cryogenic temperatures (about 90-100 K) from shock frozen crystals, immersed in very small amounts of solvents, which, upon cooling, solidify as amorphous materials (Yonath et al., 1988; Hope. et al., 1989; von Böhlen et al., 1991). An experimental procedure was designed to accommodate the unique features of the ribosomal crystals: anisotropic morphology (at least one very thin dimension), extreme softness and high fragility. Prior to cooling, the crystals are transferred to media similar to their original stabilizing solutions, but with somewhat higher viscosity. Often a cryosolvent has to be added to avoid the formation of ice crystals. A thorough empirical search to establish individual pre-cooling treatment for each crystal type was essential. The variables being the type and compositions of the added materials, the fashion of addition and the time course of the treatment (Hope et al., 1989).

All ribosomal crystals obtained so far are soft, flexible and easily deformed. When mounted on glass fibers, as is routinely done for cryo-temperature data collection of crystals of non-biological compounds or for average-size proteins, they bend around the glass fiber and loose their internal order. To suit this we constructed a variety of microspatula which allow mounting crystals in desired orientations. The more elaborate ones are made of double layers of thin glass plates ("double-layer" or "sandwich" spatula). These provide extra protection from drying and from bending stresses resulting from surface tension effects, created upon pulling crystals from the pre-cooling treated stabilizing solution.

Best results were obtained from crystals, immersed in their specific pre-cooling solutions and plunged into liquid propane near its solidifying temperature. A special apparatus was constructed, allowing transfer of the cooled crystals from the propane to the X-ray camera, where they are surrounded by a nitrogen gas stream at cryo-temperature throughout data collection. Under these conditions irradiated frozen ribosomal crystals show no signs of decay over periods longer than the time needed to collect a complete diffraction data set (days or weeks). To resume interrupted diffraction experiments, we constructed a device for preserving irradiated crystals in solid propane/liquid nitrogen chambers for extremely long periods, even a few years. To estimate the influence of the storage at cryo-temperature, the diffraction data of a fresh crystal was compared with those collected after 24 hours of irradiation and after 153 days of storage in solid propane. In both, no intensity changes were detectable.

II. The effect of shock freezing on the cell dimensions

For a large number of crystals of biological macromolecule it was shown that the shock cooling treatment effects the unit cell dimensions. In some cases, where these values could be determined at both ambient and cryo-temperature, significant shrinkage, 3-20% of the original length was reported (Shakked et al., 1990).

As most of our serious data collection experiments were carried out with synchrotron radiation at cryo-temperatures, accurate determination of the cell dimensions was not possible. Only a few

months ago were we able to determine the cell dimensions at ambient temperature, from limited data collected to around 10-12 Å, from one crystal of the large ribosomal subunits from Haloarcula marismortui, using the GX21 rotating anode coupled with an imaging plate. In this crystal form (space group $C222_1$), the a and the b axes (214 Å and 303 Å respectively) were found to be quite conserved in most of the so far measured crystal, whereas the c axis (around 570 Å), show some variability. Thus, variations of up to 1.5% were commonly observed for it between the frozen crystals.

Comparison of the cell dimensions determined at ambient-temperature with the average values obtained from the shock-frozen crystals by synchrotron radiation, showed shrinkage of up to 2.3% of the a and b axes. The ambient temperature c axis was found to be at the upper level of the average length found for it in frozen crystals. Therefore accurate analysis of the susceptibility of the c axis to the shock cooling procedure is still not possible.

A word of caution is due. These values should be considered as most preliminary, since the ambient temperature cell dimensions were determined from only one crystal and since a change of 1.5% of the c axis amounts to a change of 9 Å, which reaches the order of magnitude of the radius of globular proteins of a molecular weight comparable to that of average ribosomal proteins.

III. The reproducibility of the Freezing Procedures

Previous experience showed that even crystals grown under the same conditions from the same ribosomal preparation may show some variability in the cell dimensions. It was not clear whether this variability is an inherent property or induced by the cooling, since the rather lengthy pre-cooling treatment and the shock freezing, coupled with the inherent flexibility of the ribosome may result in gross structural variability or in apparent changes in cell dimensions.

To address this point, we halved a relatively large crystal of the 50S subunits from H. marismortui, and shocked cooled each of its halves separately. The two halves were positioned in the beam in a similar orientation, and data were collected around the cell axes of each part (12° around the a and b axes of each half). Differences of magnitude (0.25%) similar to the experimental errors in the determination of all cell axes were observed. Therefore it was concluded, that at least for this particular crystal, the shock cooling did not introduce changes. Thus, this preliminary experiment show that the process of shock cooling is rather reproducible, and if indeed it introduces shrinkage or other changes, these are of a similar magnitude in different experiments.

We plan to verify this result by repeating the experiment using crystals of different sizes, cut to even as well as non-even fragments. Unfortunately, the cutting procedure has still to be refined. Currently most of the segmented crystals are somewhat damaged presumably due to the development and propagation of strain caused by the segmentation, although we are experimenting under very fine and mild conditions.

No matter what is the source of the real, or apparent non-isomorphism of the ribosomal crystals, its existence may complicate phasing by methods which rely on difference maps (such as MIR and SIR). The cut crystals may be instrumental for overcoming these problems. Given we achieve smooth and non-damaging cutting, a procedure is currently being developed for collecting pairs (or larger number) of data sets from the same crystal. Thus, one segmented crystal should give rise to both ambient- and cryo-temperatures cell dimensions. Furthermore, for minimizing non-isomorphism effects, native data will be collected from one part of a cut crystals and the other part(s) will be measured after being soaked in solutions of potential heavy atom derivatives as well as of different densities which may facilitate phasing by contrast variation.

IV. The effect of shock freezing on resolution and mosaicity

The significant heterogeneity of the ribosomal crystals makes a quantitative assessment of the effect of shock-freezing on crystal properties impossible because the same crystal can not be exposed both at ambient and at cryo-temperatures, due to the irreversibility of the effects of radiation damage. However, for ribosomal crystals, it is clear that the mosaicity of properly shock-cooled crystals is conserved throughout the shock-freezing procedure.

The resolution limits of the diffraction patterns measured at cryogenic temperatures should not be higher than those obtained at ambient temperature, since these limits reflect the intrinsic conformational heterogeneity of the crystal. On the contrary, it is likely that the various steps in the cooling procedure, with their associated thermal, osmotic and mechanical stresses, may lead to a deterioration of crystal order. However, as the thermal motion within the frozen crystals is limited, and as at cryo temperature, the crystal can be irradiated for a long time, an apparent improvement in resolution was observed, resulting from the detection of a larger number of the higher resolution weak reflections.

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