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Shaped Protein Single Crystals

BY JUAN M. GARCÍA-RUIZ AND ABEL MORENO

Laboratorio de Estudios Cristalográficos, Instituto Andaluz de Geologia Mediterránea, CSIC-Universidad de Granada, Av Fuentenueva s/n, Granada 18002, *Spain*

AND ANTONIO PARRAGA AND MIQUEL COLL

Department de Biologia Molecular i Cellular, CSIC, Jordi Girona 18–26, 08034 *Barcelona, Spain*

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Abstract

The formation of protein single crystals grown with the shape controlled by the geometry of the capillary used as a growth cell is presented. The shaped crystals show strong birefringence under crossed nicols and diffract as single crystals up to 1.74 Å.

Introduction

The high content of water trapped in the crystalline structure of macromolecular crystals makes them valuable for the study of their biochemical properties (McPherson, 1987; Blundell & Johnson, 1976). The interactions of a wide range of compounds, such as metal ions, substrates, cofactors and drugs, can be studied by measuring the diffusion and adsorption behaviour of these compounds within the macromolecular crystal lattice (Stura & Chen, 1992) and by the conformational changes arising from these effectors. One of the current problems in measuring the kinetics of these effects arises from the experimental problems encountered when trying to constrain the transport of the diffusing units to the protein crystal lattice. Consequently, the knowledge of the biological activity of protein crystals by *in situ* catalytic assays could be enhanced by using single crystals with controlled geometry.

In this paper, we study the control of nucleation density of lysozyme crystals grown by the gel-acupuncture technique under isothermal conditions. It is shown that by reducing the number of crystals in a given capillary to less than three individuals it is possible to form protein single crystals in the shape of the inner wall of the capillary tube used as the growth cell.

Experimental

The experimental set-up utilized for the growth of protein single crystals was typical of the gel-acupuncture technique (García-Ruiz, Moreno, Viedma & Coll, 1993; García-Ruiz & Moreno, 1994). The gel layer holding the capillaries was made from silica by mixing appropriate volumes of a sodium silicate (Aldrich) water solution with specific gravity 1.06 g cm^{-3} and acetic acid solution (1 *M*). The pH of the gel layer was adjusted to 6.0.

To test the influence of the starting concentration of lysozyme solution on the final number of crystals obtained in the capillary we have performed experiments for ten different concentrations in the range 10–100 mg ml^{-1}, while maintaining the starting concentration of the precipitating agent (NaCl) at 20%(*w*/*v*). Lysozyme was used as purchased from Fluka (No. 62971). The influence of the initial concentration of the precipitating agent was studied using three different values [10, 20 and 30%(*w*/*v*)], maintaining the starting protein concentration at 100 mg ml^{-1} for all three cases. The penetration length of the capillary into the gel was 7.5 mm. The solutions were prepared with deionized and twice-distilled water; no buffers were added in any case. We used hen egg-white lysozyme as purchased from Fluka (No. 62971) without further purification. Sodium azide solution was added to the precipitating agent at 0.1%(*w*/*v*). All the experiments were carried out at laboratory temperature (293 ±1 K).

In order to measure the growth rates of the crystals some of the experiments were followed by video microscopy. We used bright-field images recorded with a television camera attached to a binocular microscope provided with a ×7 objective lens. Frames were recorded for 1 s with a time lapse of 1 min. Measurement of the advance of the crystal face was made on the television screen by applying the corresponding magnification factor.

Results and discussion

As shown in Fig. 1, the number of crystals appears to fit an exponential function of the protein concentration. The data corresponds to unbuffered experiments carried out in capillaries with an inner diameter of 1.2 mm and at 20%(*w*/*v*) starting concentration of the precipitant. The influence of the initial concentration of the precipitant agent (at a constant starting protein concentration of 100 mg ml^{-1}) was studied using three different values:

10, 20 and 30%(*w/v*) and the results reveal a higher sensitivity of the crystallization behaviour on this variable. Fig. 2 shows the influence of the capillary diameter on the number of crystals obtained in the experiment for a starting protein concentration of 100 mg ml^{-1} and a starting NaCl concentration of 20%(*w/v*). Note that the number of crystals growing into the capillary increases as a function of the capillary diameter. The size of the crystals also increases with the capillary diameter but the maximum height attained in the capillaries by the crystals does not correlate clearly with this variable.

In these and previous experiments carried out using the gel-acupuncture technique we have observed that most of the grown lysozyme crystals attach to the walls of the glass capillaries. As demonstrated above, crystal nucleation can be controlled by selecting the appropriate values of the experimental parameters; it is even possible to reduce the number of growing crystals to less than three individuals. Under such conditions, it is reasonable to expect that the nuclei attached initially can grow big enough to attain the opposite wall of the capillary, thus filling the whole capillary diameter.

Guided by the above results, we have performed some experiments using sodium chloride at an initial concentration of 20%(*w/v*) and a starting protein concentration of 100 mg ml^{-1}. The protein crystals were grown inside capillary tubes with different internal diameters from 0.2 to 0.5 mm. The height of the protein solution in the capillaries was approximately 60 mm. In all cases we obtained from one to three crystals per capillary and in most cases the crystals were separated enough to grow without interference with each other. The growing crystals were observed to grow with morphology dictated by the shape of the capillary geometry, thus becoming crystalline cylinders with a diameter that fits the inner capillary section. This shaping phenomenon was observed in capillaries with circular cross section (for diameters of 0.2 and 0.5 mm) as well as in capillaries with rectangular cross section (0.4 × 4 mm). Fig. 3

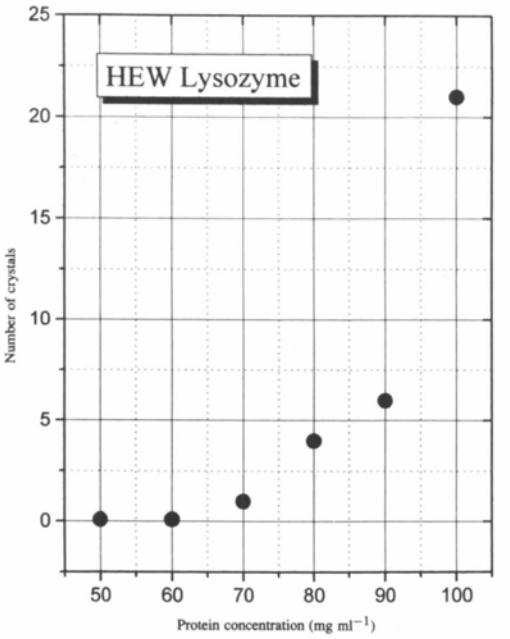

Fig. 1. The variation of the number of lysozyme crystals obtained as a function of the starting protein concentration. Starting concentration of the precipitant (NaCl), 20%(*w/v*). Penetration length, 7.5 mm; inner capillary diameter, 1.2 mm.

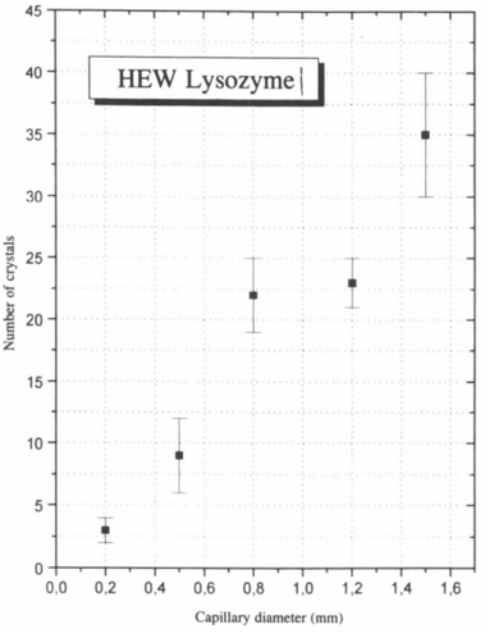

Fig. 2. The variation of the number of crytals obtained as a function of the inner capillary diameter. Starting protein concentration, 100 mg ml^{-1}; starting NaCl concentration, 20%(*w/v*); penetration length, 7.5 mm.

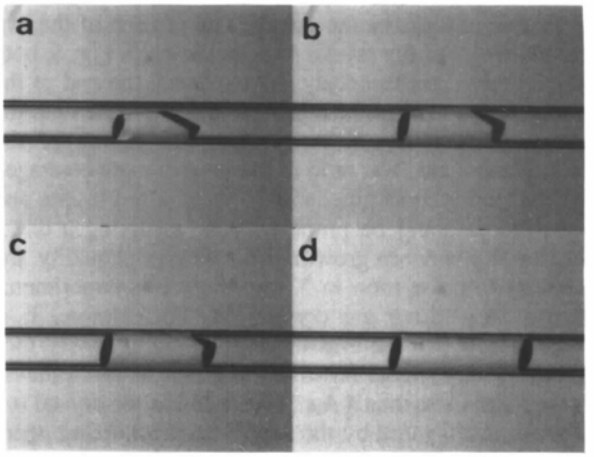

Fig. 3. Several stages of the history of a protein cylinder growing into the capillary volume. (*a*) 24 h; (*b*) 48 h; (*c*) 60 h; (*d*) 96 h. The diameter of the capillary is 0.2 mm.

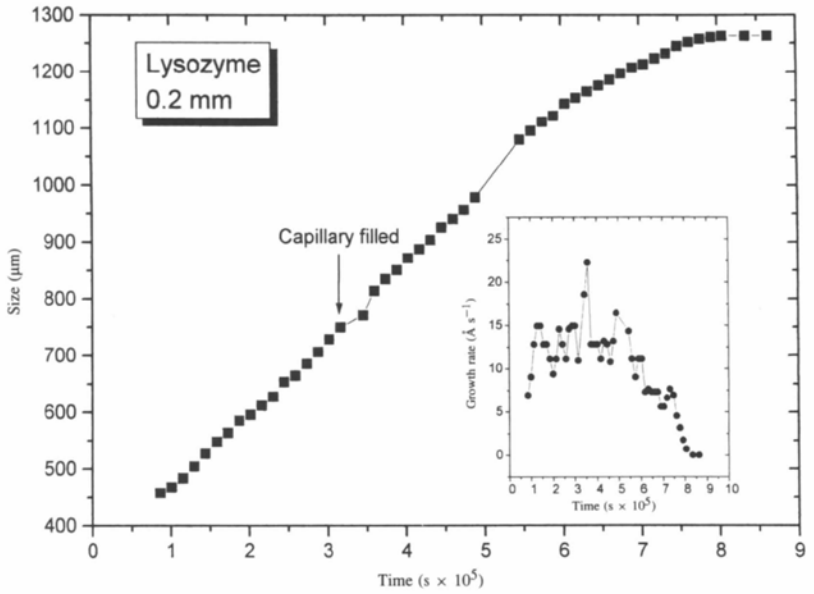

Fig. 4. Variation of the length of the cylindrical crystal with respect to time.

illustrates some frames from the film recorded by time-lapse video microscopy and Fig. 4 shows the variation of the length of the crystals as a function of time.

As shown in Fig. 3, the crystalline cylinders behave optically as single crystals when observed under crossed Nicols. To test the crystal quality, X-ray diffraction measurements were taken using the capillary glass in which the crystal grew. Crystals were kept at room temperature. As shown in Fig. 5, the protein rods diffract as a single crystal of tetragonal lysozyme. This 1° rotation photograph is of a crystal rod 1.3 mm in length which grows inside an X-ray capillary with an inner diameter of 0.5 mm. It was collected with an imaging-plate detector set at 68 mm from the crystal using a rotating anode as an X-ray source. Note that the crystal diffracts to a resolution better than 1.8 Å.

By using a reference point external to the crystal we were able to measure the growth rate of each of the two growth fronts of the crystal rod. As shown in Fig. 6, both fronts grow simultaneously. As expected, the end of the crystal facing the gradient grew at a higher rate than the surface of the crystal away from the source of the precipitant agent. The ratio of the growth rates (averaged for the total growth time) was close to seven to one and therefore, it could be proposed that, assuming a direct relationship between growth rates and crystal quality, the intensity-to-noise ratio in X-ray diffraction experiments should vary along the crystal rod. Furthermore, it is worth noting that the measured growth rates for the face of the crystal cylinder far from the source of precipitating agent was of less than 4 Å s^{-1}. This end of the crystal rod is growing triggered by the supply of precipitaing agent through the porous structure of the crystal and, therefore, is expected to have the lower growth rate. It has to be noted that the crystallographic orientation of the rods in the capillaries varies from one experiment to another which suggests that the crystal orientation is not chemically controlled by the surface of the capillary glass and, therefore, led us to expect that other proteins might also be grown as shaped single crystals.

We have been able to obtain crystal single rods up to 1350 μm in length and 0.2 mm in diameter and crystals up to 1400 μm with 0.5 mm diameter. The height of the capillary column of protein solution was 60 mm and, therefore, the volume of protein solution contained in the capillary was 1.88 μl for capillaries 0.2 mm in diameter. As the concentration of protein in the capillary was

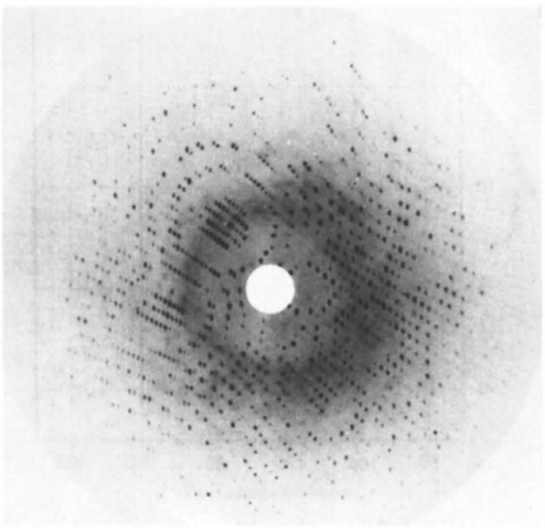

Fig. 5. X-ray rotation image of a protein cylinder showing its single-crystal character. Maximum resolution is 1.74 Å.

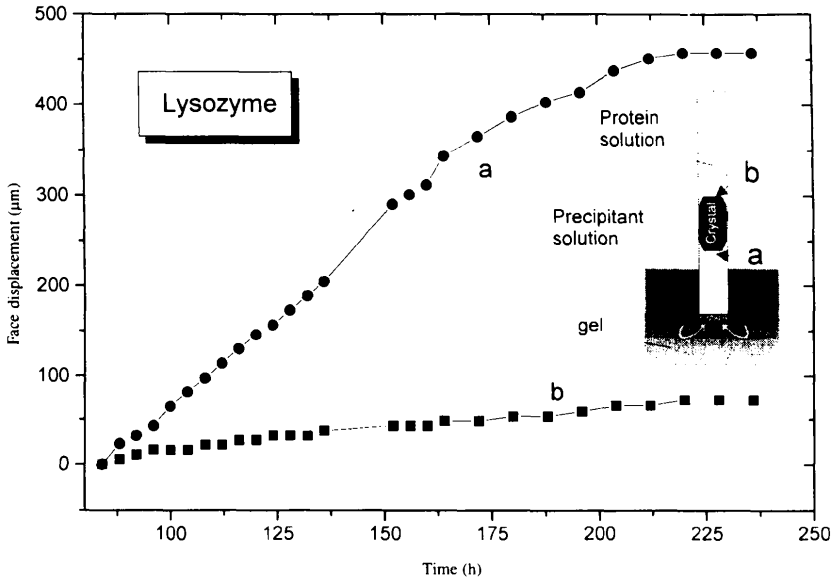

Fig. 6. The advance of the two faces of the cylindrical crystal in time. Time scale starts when the experiment is implemented and the first data corresponds to the point when the width of the protein crystal equals the inner capillary diameter.

100 mg ml^{-1}, the total mass of protein in the starting solution was 0.18 mg. Lysozyme density is known to be 1.23 g cm^{-3} (Privalov, Tiktopulo, Venyaminov, Griko, Makhadatze & Khechinashvili, 1989) and, therefore, the protein consumed by the growth of the cylindrical crystal can be calculated to be 0.052 mg. In terms of efficiency this means that more than 25% of the protein in the mother solution is sequestered in the form of a single crystal.

For those proteins displaying a significant variation of solubility with temperature, and a wide metastable zone, it should be possible to produce the shaping phenomenon as described above by using a polythermal growth method. Possibly, the experiment could be performed in a way similar to the one currently used to obtain cylindrical single crystals by the floating-zone method in melt growth (see for instance, Feigelson, 1989). In spite of the fact that it is theoretically possible, the main disadvantage arises from the microscale required in the case of protein crystallization which makes it technically difficult to control a sharp temperature gradient.

Protein single crystals of well defined and controlled shape have advantages for X-ray absorption corrections but the main application could be their use in binding studies as they completely fill the geometry of the diffusion cell providing a precise reference framework for kinetic studies. The development, in time, of binding processes could be in the future followed by *in situ* X-ray diffraction, calorimetry and other techniques. Moreover, the possibility of growing these shaped crystals leads the way to applications such as the use of protein crystals as molecular sieves. The precipitation of inorganic nanophases inside a protein single crystal by counterdiffusion of reactants through the macromolecular crystal is another plausible application as these have already been obtained from protein solutions (Mann & Meldrum, 1991). Based on our previous data on the growth of PbS in gels (García-Ruiz, 1986), longer protein rods will be required. Our preliminary results indicate the possibility of growing larger shaped crystals by the method described above. For instance, the largest crystal we have obtained which filled a capillary with rectangular cross section was 1.9 × 4 × 0.4 mm. The crystal size could be optimized by maintaining the same starting protein concentration but using longer capillary lengths. We are currently performing experiments to produce larger shaped crystals of lysozyme by using the crystals described above as seed.

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