Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystal-growth kinetics of protein single crystals along capillary tubes in the gel-acupuncture technique

Abel Moreno* and Manuel Soriano-García

Departamento de Bioquímica, Instituto de Química, UNAM Circuito Exterior, CU México, DF 04510, Mexico

Correspondence e-mail: carcamo@servidor.unam.mx

Received 3 November 1997
Accepted 26 October 1998

In attempts to obtain protein crystals of a sufficient size for structural studies, lack of knowledge of the physicochemical properties of protein solutions and of their crystal-growth behaviour lead to a bottleneck for drug design as well as for X-ray crystallography. Most formal investigations on crystal-growth phenomena have been focused on equilibrium studies, where the protein is soluble, and on the kinetics of crystal growth, which is related to both nucleation and crystal-growth phenomena. The aim of this work is to measure the crystal-growth rate along a capillary tube used as a growing cell. These experiments were carried out using the gel-acupuncture technique [García-Ruiz *et al.* (1993). *Mater. Res. Bull.* **28**, 541–546; García-Ruiz & Moreno (1994). *Acta Cryst.* D**50**, 484–490; García-Ruiz & Moreno (1997). *J. Cryst. Growth*, **178**, 393–401]. Crystal-growth investigations took place using lysozyme and thaumatin I as standard proteins. The maximum average growth rate obtained in the lower part of the capillary tube was about 35 Å s^{-1} and the minimum average growing rate in the upper part of the capillary tube was about 8 Å s^{-1}. The crystal-growth rate as a function of the supersaturation was experimentally estimated at a constant height along the capillary tube.

1. Introduction

Crystal-growth equilibria is, in a sense, a contradictory definition, as crystals do not grow at equilibrium. Nevertheless, it is essential to have some understanding of the equilibria involved before studying the kinetics of any process. The steady state can shed considerable light on the growth equilibria and lead to the crystal-growth process. Additionally, several equilibria parameters such as temperature, pressure and concentrations of reactants can be varied and these may promote the crystal growth. This enables us to simplify the classification of the growth process.

Crystal growth is a heterogeneous chemical reaction of one of the following types: (i) solid to crystal, (ii) liquid to crystal or (iii) gas to crystal. Crystal growth may take place in a system where, except for traces of impurities or low concentrations of deliberately added dopants, the only component present is the material which is being crystallized. We shall call growth under these conditions 'monocomponent crystallization'. Growth may also take place in a system where the impurity concentration or the level of the added dopant is high. In this case, the material to be crystallized is either dissolved in a solvent or formed by means of a chemical reaction. Such growth is taking place in a system in which one or more additional components to the component which is forming the crystal are present, and we shall call this 'polycomponent crystallization'.

Kinetics deals with the rate of a process or a reaction, taking into account all factors that influence the rate of the process, as well as explaining the rate either in terms of the reaction path or the reaction mechanism. For a crystal to grow, as for a chemical reaction to take place, a number of steps must be followed. If one of these steps is slower than the others, then the observed rate will be determined by the rate of this rate-limiting step. If several steps are of comparable rates, then the observed rate will depend on the rates of all these steps, and the reaction is known as a consecutive reaction. For crystal growth, the rate will depend on these processes and on the conditions employed for growing crystals.

Most investigations focus on growth-rate determination based on classical techniques (hanging-drop, sitting-drop and batch methods) published elsewhere (see references in García-Ruiz & Moreno, 1997). It is clear that the crystal-growth technique is important for obtaining crystal-growth rates in order to produce high-quality crystals and in maintaining the same diffusion path throughout the experiment. For the gel-acupuncture technique, as for any spatially heterogeneous open system moving towards equilibrium, the environmental supersaturation at the location

Table 1
Crystallization conditions using the gel-acupuncture technique.

All experiments were performed at 291 K.

Protein (mg ml^{-1})	Buffer	Precipitating agent
Lysozyme (100)	Sodium acetate 100 m*M* pH 4.6	NaCl 20%(*w*/*v*)
Thaumatin I	Phosphate 100 m*M* pH 7.0	Potassium/sodium tartrate 20%(*w*/*v*)

where the crystal grows is time dependent. A high-accuracy measurement of the exact local supersaturation, in the light of present knowledge of diffusion reaction systems, is a real problem. Several approaches have been used to attempt to calculate the growth rate with an acceptable accuracy by using interferometric techniques. Thus, the concentration variations of the protein solution as well as the mass gradients towards crystal growth have been well established (Cole *et al.*, 1995; Kuznetsov *et al.*, 1996).

2. Materials and methods

Fig. 1 shows a diagram of a glass cassette used for monitoring the growth rates of crystals inside the capillary tubes and obtaining growth-rate data. It consists of two rectangular glass plates of 70 × 100 mm held apart by a U-shaped spacer covering the two longest sides and one of the shorter sides of the plates. After fastening the plates with clamps, the gap left between them is approximately 4 mm wide. This is then filled to a height of 40 mm with 8 cm^3 of a silica sol. Once this has gelled, the capillaries containing the protein solution are punctuated into the gel with a penetration length of 7 mm. Finally, 8 cm^3 of the precipitating solution is poured over the gel (details of gel preparation have been previously published; García-Ruiz *et al.*, 1998; Moreno *et al.*, 1999). The uncovered shorter edge of the cassette is then closed with adhesive tape and the cassette is placed under an optical microscope. The cassettes are then aligned perpendicular to the gravity field. A television camera attached to the microscope captures the image which is sent to a video recorder provided with a time-lapse mechanism. For these particular experiments, a video image is recorded every minute.

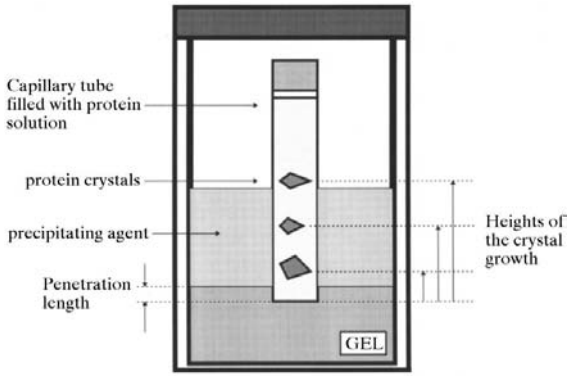

Figure 1
The experimental setup of the gel-acupuncture technique for crystal-growth kinetics measurements along the capillary tube. The internal diameter of capillary tube was 0.5 mm.

We used cylindrical capillaries with an internal diameter of 0.5 mm. In addition, some experiments were also performed using capillaries with a 4 × 0.4 mm rectangular cross section. Prior to the experiment, the capillaries were washed with double-distilled water and acetone and dried with air. Thaumatin I (Sigma, T-7638) and lysozyme (Sigma, L7773) were used as purchased without further purification. Sodium potassium tartrate and sodium chloride were used as precipitating agent solutions, respectively. All the solutions were prepared with double-distilled water. The layer holding the capillaries was comprised of silica synthesised by mixing the appropriate volumes of sodium silicate solution with a specific gravity of 1.06 g cm^3 and 1 *M* acid. The pH of the gel was 6.5 for thaumatin and 4.5 for lysozyme. For both thaumatin and lysozyme, phosphate and acetate buffers were used to maintain the protein solutions at pH 7.0 and 4.5. 0.1%(*w*/*v*) sodium azide solution was added to the precipitating agent. A summary of the crystallization conditions for lysozyme and thaumatin is given in Table 1.

The crystals were monitored for several days. The growth rates were obtained by measuring the linear size of the crystal on the video screen and converting the values obtained to the actual size using a previously calculated calibration factor. As discussed in our previous publication (García-Ruiz & Moreno, 1997), all crystals studied grew in an euhedric habit and remained similar in shape throughout the growth process.

In addition, we monitored the growth rate along the capillary tube as a function of the supersaturation ($\beta = C/C_{\rm equilibrium}$). We estimated the concentration of the protein (steady state) after crystal growth had finished ($C_{\rm equilibrium}$) after waiting for a week in the case of lysozyme and two weeks for thaumatin. The initial concentration (C) and $C_{\rm equilibrium}$ were used to calculate the starting supersaturation $C/C_{\rm equilibrium}$ for each experiment. $C_{\rm equilibrium}$ for thaumatin and lysozyme was 44 and 34 mg ml^{-1}, respectively.

Table 2
Growth rates of thaumatin and lysozyme along the capillary tube using the gel-acupuncture technique.

Protein	Height of crystal growth (mm)	Growth rate (Å s^{-1})
Thaumatin	3.0	35.00
	4.5	9.55
	25.0	8.32
Lyzozyme	4.0	35.0
	20.0	8.21

3. Results and discussion

Previous work describing continuous video recording of the growth of single protein crystals under isothermal conditions at constant supersaturation has been published elsewhere (Koszelak & McPherson, 1988, and references therein). Although we performed most of the measurements using classical vapour-diffusion methods (hanging and sitting drop), we have also measured the crystal-growth rate in capillary tubes. In this experimental setup the capillaries act as growing cells. The diffusive transport of the precipitating agent is the rate-limiting step, as has been shown by spectroscopic analysis (García-Ruiz & Moreno, 1997). Therefore, the aim of this article is to complete our previous publication on the kinetics of crystal growth of protein single crystals, where almost all the growth rates were evaluated, with the exception of the growth rate along the capillary tube. All these experiments were based on the gel-acupuncture experimental setup previously mentioned. Here, we have determined the growth kinetics of proteins along the capillary tube as a function of the height of the capillary tube and the overall supersaturation of the system. According to the experimental results, the growth rates in the upper part of the capillary tube (8.32 Å s^{-1}, for thaumatin I and 8.21 Å s^{-1} for lysozyme) were smaller than those in the lower part (35 Å s^{-1} in both cases). At the beginning of the experiment, it was noticed that the growth rate for lysozyme in the lower part of the capillary tube was higher than that in the upper part (see Table 2). This observation made us consider using thaumatin I as a

model protein in order to test the crystal-growth rates along some established capillary-tube heights (Table 2). Once the video-microscopy measurements have been performed, the growth rate seems to decrease, as was also observed with lysozyme in the same capillary tubes.

The explanation of this phenomenon is based on the existence of high values of supersaturation and the subsequent relaxation of the supersaturated system towards equilibrium. In all these experiments, the supersaturation was stable in the initial stages of crystallization, keeping the normal growth rates virtually constant. The normal growth rate G (R) eventually decreases with time owing to the depletion of the protein solution. This is crucial in understanding how the gel-acupuncture method of protein crystallization works and why it is that a higher quality of crystals is obtained in the upper part of the capillary tube. In our experimental setup, the residence time of a crystal growing on the supersaturating wave in the upper part of the capillary is higher than that of a crystal in the lower part of the capillary.

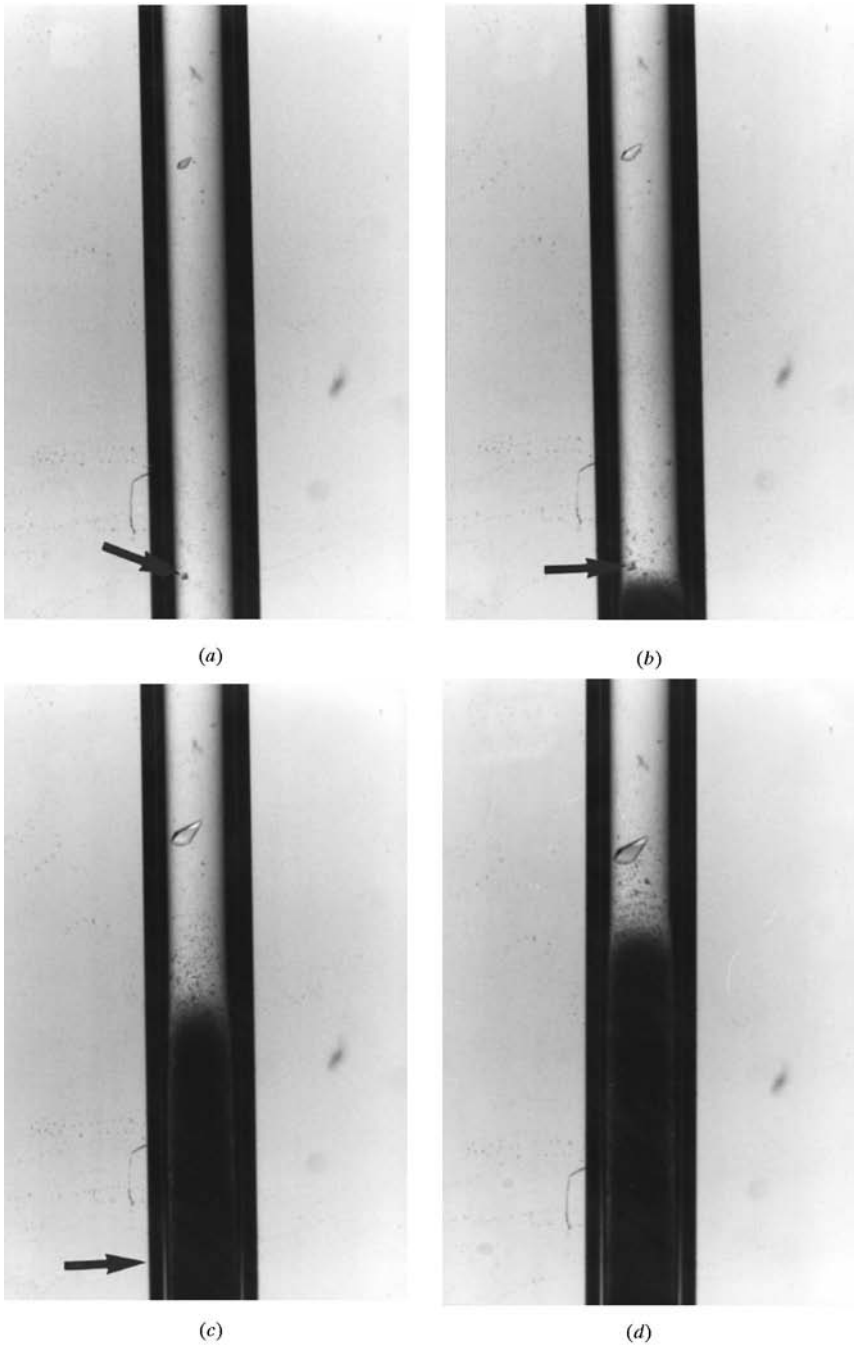

Figure 2
Sequence of photographs of lysozyme crystal growth in capillary tubes at higher supersaturation. (*a*) $t_0 = 0$, (*b*) t = 240 min, (*c*) t = 660 min and (*d*) t = 870 min.

Table 3
Growth rate of lysozyme as a function of supersaturation.

The height where the crystal appeared was 20 mm.

Growth rate (Å s^{-1})	Supersaturation
8.21	3.00
10.40	5.88

A good approach is to use a computer simulation for predicting the growth rates along the capillaries (Otálora & García-Ruiz, 1996). In our case, the growth rate was experimentally measured as a function of the supersaturation. For each experiment, once the first crystal appeared inside the capillary tube at a certain height, its growth rate was measured and recorded by the video-microscopy technique without any disturbance of the crystal-growing cell. The average growth rate was then estimated using a calibration scale. According to these experimental results, the crystal-growth rate increased as the supersaturation of the system increased. It was also observed that lower rates were obtained at increasing heights along the capillary tube. In order to demonstrate this crystal-growth behaviour, the growth rates were measured at a constant height for two different values of supersaturation for lysozyme (3.00 and 5.88) and three different values of supersaturation (2.27, 3.41 and 4.55) for thaumatin, as shown in Tables 3 and 4, respectively.

Based on the theories of Burton–Cabrera–Frank (BCF) and Kossel–Stranski (KS), we came to the conclusion that it was important not only to estimate the growth rate, but also to evaluate the mechanism of crystal growth. At higher supersaturation the morphology of the crystal was not modified, as predicted by the BCF and KS theories. In all the experiments, the crystals at high supersaturation were slightly larger in size than those obtained at lower supersaturation. This trend may occur as a consequence of some protein being present as a precipitate and promoting the crystal growth. At the beginning of the lysozyme crystallization experiment, a precipitate was obtained in the lower part of the capillary prior to the appearance of the first crystals. Subsequently, this precipitate dissolved and the crystals grew along the capillary tube. The precipitate acts as a source of protein molecules for crystal growth. In the lysozyme case, there is a problem measuring the complete growth at certain heights at the

Table 4
Growth rate of thaumatin as a function of the supersaturation.

The height where the crystal appeared was 4.5 mm.

Growth rate (Å s^{-1})	Supersaturation
9.55	2.27
12.50	3.41
67.50	4.55

highest supersaturation. As is observed in the sequence of photographs showing lysozyme crystal growth in real time (Figs. 2*a*–2*d*), the amorphous precipitate can obscure a crystal which is located at a lower height (Fig. 2*a*; the arrow shows a small crystal subsequently obscured by the amorphous precipitate); hence, some growth rates in the lower part of the capillary tube are impossible to measure. A physicochemical explanation for the appearance of this amorphous precipitate prior to lysozyme crystal growth is in progress.

4. Concluding remarks

The transportation of the precipitating agent takes place by diffusion in capillary tubes with an internal diameter of less than 1.0 mm. This trend has previously been determined using spectroscopic techniques (García-Ruiz & Moreno, 1997). The crystal-growth rate is a function of the crystal location in the capillary tube and of the supersaturation. The minimum average growth rate was about 8 Å s^{-1} in the upper part of the capillary, explaining the good quality of the crystals observed for the proteins studied. Although this article experimentally shows different rates measured at specific heights of the capillary, it is important to bear in mind that the rate-limiting step is a diffusion-controlled phenomena (García-Ruiz & Moreno, 1997). These results take into account the size of the capillary tube as well as its thickness in order to determine the internal diameter, the magnitude of which can cause the diffusion path to be stopped or maintained under the same crystallization conditions.

We believe that growing crystals in capillary tubes could be particularly useful for obtaining larger protein crystals. Using this approach we can not only determine a way to control the convection, but also a way to control the supersaturation wave as it travels along the capillary length. Therefore, the crystallization of proteins will be both easier and more rapid. A possible way of achieving this is by varying the initial conditions so that the speed of the supersaturating wave is slowed in order to match the rate of the crystal growth. In theory, the result could be crystals stretching from the point of initial nucleation to the end of the capillary tube. Although it is possible to obtain high-quality crystals using the gel-acupuncture technique, our goal for the future is to control the supersaturating wave in order to obtain larger crystals.

The authors acknowledge financial support from Direccion General de Asuntos del Personal Academico (DGAPA-UNAM) projects number IN218597 and IN221398. This is contribution number 1672 from the Instituto de Química, UNAM.

References

Cole, T., Kathman, A., Kozselak, S. & McPherson, A. (1995). *Anal. Biochem.* **231**, 92–98.
García-Ruiz, J. M. & Moreno, A. (1994). *Acta Cryst.* D**50**, 484–490.
García-Ruiz, J. M. & Moreno, A. (1997). *J. Cryst. Growth*, **178**, 393–401.
García-Ruiz, J. M., Moreno, A., Rondon, D., Otalora, F. & Zauscher, F. (1998). *J. Chem. Ed.* **75**, 442–448.
García-Ruiz, J. M., Moreno, A., Viedma, C. & Collect, M. (1993). *Mater. Res. Bull.* **28**, 541–546.
Koszelak, S. & McPherson, A. (1988). *J. Cryst. Growth*, **90**, 340–345.
Kuznetsov, Yu. G., Malkin, A. J., Greenwood, A. & McPherson, A. (1996). *J. Cryst. Growth*, **166**, 913–918.
Moreno, A., Juárez-Martínez, G., Hernández-Pérez, T., Batina, N., Mundo, M. & McPherson, A. (1999). *J. Cryst. Growth.* Submitted.
Otálora, F. & García-Ruiz, J. M. (1996). *J. Cryst. Growth*, **169**, 361–367.