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An investigation of magnetic field effects on the dissolution of lysozyme crystal and related phenomena

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It is now widely known that a magnetic field, either homogeneous or inhomogeneous, depresses the growth process of protein crystals. In this report, the dissolution process of tetragonal lysozyme crystals is also confirmed to be depressed by a homogeneous magnetic field (inhomogeneity <1.5%). The dissolution process was monitored using a Mach–Zehnder interferometer. The results showed that the concentration change during the dissolution process was slowed in a magnetic field compared with that in the absence of a magnetic field. It was concluded that the diffusion coefficient of the lysozyme molecules in the solution was decreased by the magnetic field. The decrease in the diffusion coefficient may contribute to the slowed growth process. The changes in the spatial concentration distribution under a vertical temperature gradient before crystallization in the absence of a magnetic field was also studied. The concentration in the lower, colder part of the cell increased, while it decreased in the upper, hotter part, a similar phenomenon to that discovered by previous investigators in an isothermal supersaturated solution system. Aggregated domain formation is proposed to explain the concentration redistribution before crystal growth and a suspended crystal model is proposed to explain the decrease of diffusivity in a magnetic field.

1. Introduction

Preparation of high-quality protein crystals is a challenging goal for scientists working in the field of protein crystal growth. Many efforts have been made over the past years. For instance, scientists have tried to grow protein crystals in a microgravity environment (McPherson, 1997; Garcia-Ruiz, 1998; Berisio *et al.*, 2000). Other methods, including protein crystal growth in a gel (Lopez-Jaramillo *et al.*, 2001) or in a magnetic field (Astier *et al.*, 1998; Sakurazawa *et al.*, 1999; Wakayama, 1998; Yanagiya *et al.*, 2000), have also been tried. As reported recently by Lin *et al.* (2000) and Sato *et al.* (2000), it has been found that protein crystals of better quality can be obtained using either an inhomogeneous or homogeneous magnetic field compared with those crystals prepared under the same conditions but in the absence of a magnetic field. Magnetic fields have been found to depress lysozyme protein crystal growth (Yanagiya *et al.*, 2000; Yin *et al.*, 2001) and the dissolution process (Yanagiya *et al.*, 2000). As both experimental results and theoretical predictions of convection behaviour in protein solution in a homogenous magnetic field indicate the damping of convection by a homogeneous magnetic field to be negligible (Yin *et al.*, 2001), it is hard to

explain why the growth process of protein crystals is depressed in a homogeneous magnetic field. Recently, Haik *et al.* (2001) have found that the apparent viscosity of blood is decreased by a strong magnetic field, Zhong & Wakayama (2001) have found that the apparent viscosity of a lysozyme protein solution increases in a magnetic field and recent birefringence experiments have implied that a magnetic field might cause an ordered structure along the magnetic field lines (Zhong *et al.*, 2001). These results might provide useful information for understanding the effects of a magnetic field on protein crystal growth. However, more work is still necessary as the mechanisms of the effects of a magnetic field on protein crystal growth are not yet fully understood.

Dissolution of protein crystals, in common with their growth process, is closely related to the processes occurring in the solution environment. The effect of a magnetic field on both processes should be closely related. Therefore, it is rational to study the effect of the magnetic field on the dissolution process of protein crystals more fully, which should be very helpful in clarifying the mechanisms of the magnetic field effect on protein solutions and/or the protein crystal growth process. In this report, we studied the dissolution process of lysozyme crystals in a homogeneous magnetic field using a Mach–Zehnder interferometer. The concentrations at various positions in the cell were monitored during the growth and dissolution processes of the crystals. Under a vertical temperature gradient, a concentration redistribution in the solution was found in the growth process, which may be correlated to the formation of small aggregates and/or crystals. The mechanism is believed to be similar to that for the effects observed in an aged supersaturated solution under isothermal conditions by previous investigators (Mullin & Leci, 1969; Larson & Garside, 1986; Myerson & Lo, 1991; Ohgaki *et al.*, 1991). The dissolution process was shown to be depressed by the magnetic field and the diffusion coefficient in a magnetic field was deduced from the dissolution experiment. A possible model will be proposed to explain the magnetic field effect on the diffusion coefficient.

2. Experimental

In the current study, we used the same experimental setups as described elsewhere (Yin *et al.*, 2001), except that the sample holder was equipped with water-cooling jackets on both the upper and lower sides, so that the temperature on both sides can be controlled between 273 and 333 K. Either temperature gradients or isothermal processes can be realised with high temperature-control accuracy (to ± 0.1 K) using this sample holder.

In this experiment, the sample cell was placed at the centre of a superconducting magnet (TM-6VH30; Toshiba Co.) so that a highly homogeneous magnetic field can be applied to the sample. The inhomogeneity of the magnetic field, $\Delta B/B$, was less than 1.5% [where ΔB is the largest deviation of the magnetic induction within the defined cylindrical space 50 (diameter) $\times$ 20 mm and B is the magnetic induction at the centre of the magnetic bore]. The inner thickness of the sample quartz cell was 1 mm, the height 15 mm and the width 26 mm.

The starting aqueous protein solution consisted of 45 mg ml^{-1} hen egg-white lysozyme (HEWL; Seikagaku kogyo, six times recrystallized) and 40 mg ml^{-1} NaCl; the pH of the solution was adjusted to 4.60 using 1 *M* HCl solution.

The concentration was measured *in situ* based on the relationship of the refractive index and the protein concentration in the solution under different temperatures at a certain point (x, y),

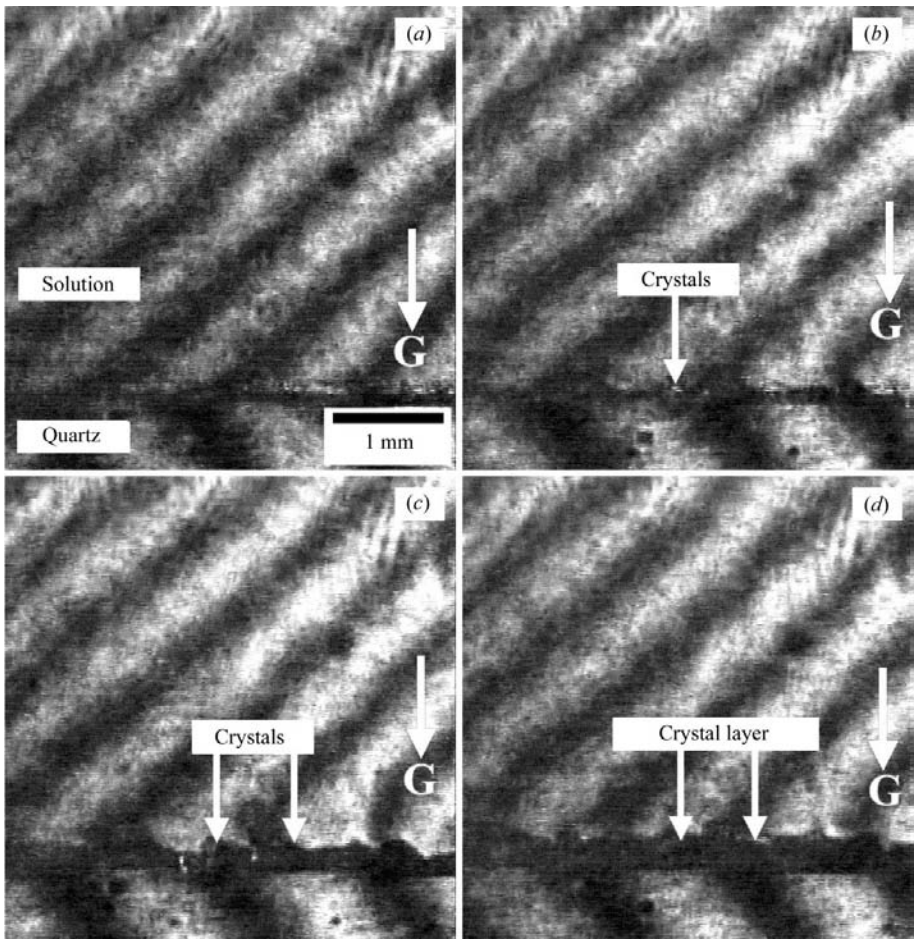

Figure 1
Growth of the layer of lysozyme tetragonal crystals. (*a*) The fringe image of the as-prepared solution; (*b*) the fringe image soon after the onset of crystal growth; (*c*) and (*d*) the fringe images of the sample at intervals of 2 h after the onset of crystal growth; these crystals were grown under a temperature gradient of 2.41 K mm^{-1}. **G** is the direction of gravity.

$$\left\{\left(\frac{\partial n}{\partial C}\right)_T [C(x,y) - C_0] + \left(\frac{\partial n}{\partial T}\right)_C [T(x,y) - T_0]\right\} d = \frac{\lambda}{2\pi} \Delta\varphi(x,y), \quad (1)$$

where n is the refractive index, C is the concentration of the solution, $C(x, y)$ is the concentration at point (x, y), C_0 is the initial concentration, $T(x, y)$ is the temperature at point (x, y), T_0 is the initial temperature, d is the thickness of the solution in the direction of the optical path (here, d = 1 mm), λ is the wavelength of the optical light source (λ = 780 nm) and $\Delta\varphi(x, y)$ is the phase difference between the initial stage and studied stage at point (x, y). From the above equation, the concentration of the solution at any time and any region $C(x, y)$ can be calculated after we have obtained the data for the interference-fringe shifts, which show the phase differences over time. As described by Yin *et al.* (2001), concentration-measurement errors caused by environmental reasons were eliminated by taking account of the phase shift in the bottom quartz part of the cell, so that the calculation result is reliable. The final calculation result should have a precision within ± 0.36 mg ml^{-1} in protein concentration if we allow for a 10% error in the fringe-shift measurement, which is reasonable for manual fringe-shift measurement.

3. Experimental results

3.1. Growth of the layer of lysozyme crystals

Before dissolution, a layer of tetragonal lysozyme crystals was prepared. This was achieved by imposing a temperature gradient on the sample cell. Fig. 1 shows the interference-fringe images during the preparation of layered crystals under a temperature gradient of 2.41 K mm^{-1}. There were no visible crystals in the as-prepared solution (see Fig. 1*a*). Fig. 1(*b*) shows the image captured when crystals started to form at the cell bottom. The curvature appearing at the end of the fringes near the cell bottom indicates a concentration gradient arising from crystal formation. Figs. 1(*c*) and 1(*d*) show the growing layer at time intervals of 2 h after the onset of crystal growth. It is clear that layered crystals could be formed under a temperature-gradient controlled program.

3.2. Crystal dissolution processes: magnetic damping of the dissolution process

Fig. 2 gives the results of interferometric observations on the whole process from growth to the dissolution of the crystals both at 0 T and in a homogeneous magnetic field of 6 T. Fig. 2(*a*) shows the arrangement of the monitored points and the temperature gradient applied. Fig. 2(*b*) represents the temperature program for the whole experiment. First, we kept the temperature at the upper side of the cell at 333 K to avoid crystallization in this area; the temperature at the lower side was slowly decreased from 333 to 278 K, so that a layer of crystals could form at the bottom of the cell. The temperature at the lower side was increased to 318 K within 30 min to dissolve the crystal layer soon after the magnetic field was increased to 6 T (see Fig. 2*c*, in which the magnetic field program is illustrated). Fig. 2(*d*) gives the time course of the lysozyme concentration for the whole process. The concentrations were measured at four points in the cell (points 1–3, 0.5, 1.36 and 3 mm away from the cell bottom and at the centre

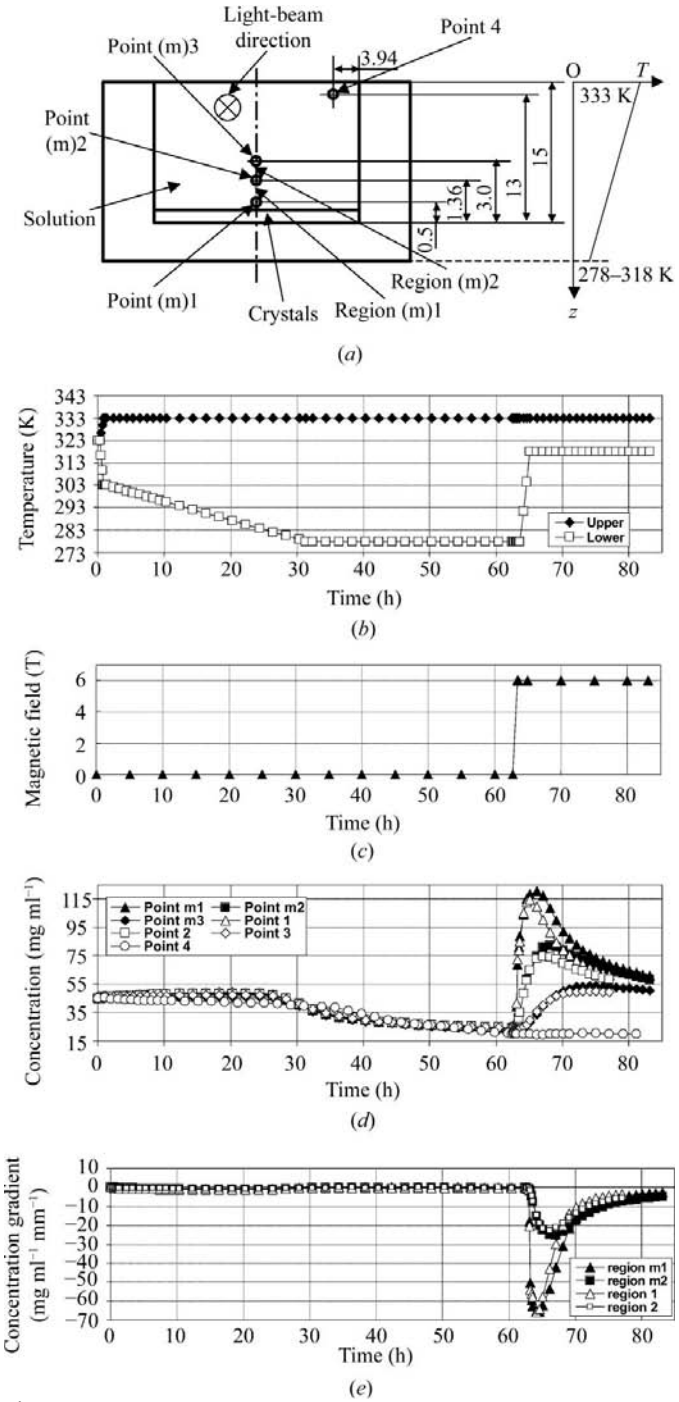

Figure 2
Experimental results for the dissolution study with and without magnetic field. (*a*) Schematical illustration of the arrangement of the points and regions for concentration monitoring; (*b*) temperature program; (*c*) magnetic field program; (*d*) time course of the lysozyme concentration at four points in the solution with and without magnetic field; (*e*) concentration gradients at two regions in the solution. Points 1–4 and regions 1–2 denote the cases without magnetic field, while points m1–m3 and regions m1–m2 denote the cases in which a magnetic field was applied in the dissolution process.

of the cell width; point 4, 13 mm away from the cell bottom and 3.94 mm away from the cell wall). Correspondingly, points m1–m3 refer to the case when a magnetic field of 6 T was applied during the crystal dissolution. The open and solid symbols represent the cases without and with the magnetic field, respectively. Concentration measurement at point 4 in a magnetic field was not possible owing to mechanical limitations on the movement of the cell in the magnet.

Comparing the concentration variations between the two conditions, we find that at the beginning of the dissolution process (about 64 h after starting the experiments) the concentration increased rapidly at points 1–3 (lower part of the cell), while at point 4 (upper part) the concentration did not change appreciably within the experimental time. After approximately 2–5 h from the beginning of the dissolution process, the concentration began to decrease. The rate of decrease at 0 T was obviously faster than that at 6 T. At point 1, the largest concentration difference between the two cases at 0 and 6 T was more than 17 mg ml^{-1}. As we know, in the case of lysozyme crystal growth under isothermal conditions the concentration-measurement results can be very scattered, probably owing to the uncertainty in the positions of nucleation and growth. The measured concentration difference between the experiments under the same controlled isothermal conditions can be more than 10 mg ml^{-1}. However, in the case of temperature-gradient-controlled lysozyme crystal growth, the concentration-measurement results showed excellent reproducibility. The statistical error in this study is as small as $\pm$0.43 mg ml^{-1}, which is much smaller than the concentration difference caused by the magnetic field during the dissolution process, as seen in Fig. 2(*d*).

According to the above experimental results, the lysozyme concentration decreased more slowly at 6 T than at 0 T. Fig. 2(*e*) shows a comparison of the concentration gradient in two regions (regions 1 and 2 for the case with no magnetic field and corresponding regions m1 and m2 for the case at 6 T, as shown in Figs. 2*a*) for the same experiment. The figure clearly shows that the concentration gradient was larger in the magnetic field. From Fig. 2(*d*) and 2(*e*), we conclude that the concentration changes more slowly in the presence of a magnetic field than in the absence of a magnetic field during the dissolution process, which implies that the dissolution process is depressed in the magnetic field. This is in agreement with the experimental observations by Yanagiya *et al.* (2000)

It is worthwhile to point out that under the experimental conditions it is possible to find orthorhombic crystals in the upper part of the cell, as the temperature and solution conditions are suitable for the growth of orthorhombic crystals. During the dissolution process of the tetragonal crystals, orthorhombic crystals continued to grow. This might affect the concentration profile in the cell. However, since the orthorhombic crystals were far from the dissolution region and the dissolution process was rather rapid, even if the magnetic field affects the growth process of orthorhombic crystals, their growth at a location far from the dissolution region will not affect the dissolution concentration differences between the two cases at 0 and 6 T. This was confirmed in a subsequent series of experiments (conducted in another magnet, JMTD-10T100M, maximum field strength 10 T; made by Japan Magnet Technology, Inc.) in which there were no orthorhombic crystals formed in the cell on changing the temperature program and solution conditions. From this series of experiments, the above results were also verified to show good reproducibility. Furthermore, the dissolution process of a layer of oriented tetragonal crystals prepared in a magnetic field of 10 T was also studied. Similar results were obtained regardless of the orientation of the crystals.

3.3. From as-prepared solution to crystal growth: concentration redistribution before crystal growth

In the above experiment, the complete process of the concentration change starting from the as-prepared solution was recorded. The concentrations at different points were found to change differently before crystal growth, although they start from the same concentration level. This attracted strong interest. By examining the concentration change in the previous 64 h in Fig. 2(*d*), it can be seen that the concentrations in the lower part of the cell seem to have increased during the first 25 h. The beginning of the concentration decrease at around 25 h apparently denotes the start of crystal growth. But before the crystal growth, does any concentration redistribution occur? To examine the concentration distribution in the cell, we measured the concentration in the upper part of the cell (point 4) for the same experimental conditions. For clarity, the results were redrawn in Fig. 3 for points 1 and 4 as the lower and upper part concentrations, respectively. It can clearly be seen that the concentration started to decrease in the upper part of the cell and to increase in the lower part soon after the solution was prepared. The largest concentration difference was more than 7 mg ml^{-1} at about 21 h. Such a concentration redistribution should be the same as the density gradient appearing in the gravitational field that is reported in the literature (Mullin & Leci, 1969; Larson & Garside, 1986; Myerson & Lo, 1991; Ohgaki *et al.*, 1991) as being found in aged supersaturated solutions under isothermal conditions,

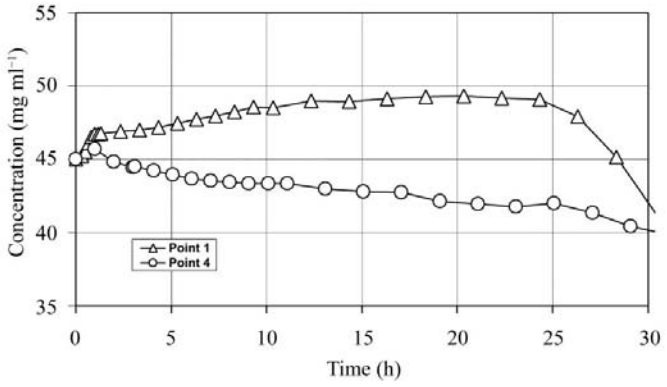

Figure 3
Comparison of the concentration changes before crystal growth in the upper part (point 4) and the lower part (point 1) of the cell (note there is no magnetic field applied). The concentration tends to increase in the lower part of the cell, while in the upper part it tends to decrease.

mostly with inorganic solution systems. This will be discussed in the following section.

4. Discussions

In the present experiment, the dissolution of the tetragonal lysozyme crystals was basically a diffusion-dominated process owing to the inner dimensions of the sample cell and the vertical temperature gradient used (Fig. 2*a*). Therefore, the depressed dissolution process implies that the diffusion coefficient of the protein molecules in the solution was decreased by the magnetic field. This simple, direct and qualitative result provides a partial explanation of our previous experimental results on protein crystal growth (Yin *et al.*, 2001), which pointed out that the magnetic field slowed down the growth process for the same cell configuration. In the growth experiment, the mass transport in the growth process is controlled by the convection near the liquid–crystal interface and the diffusion in the bulk solution. If we suppose that the convection was not influenced by the magnetic field, as we pointed out in the previous paper, the slowed growth process also indicated that the diffusion in the solution was decreased under the magnetic field. This is in good agreement with our current result.

It is very interesting to study why the magnetic field showed such a damping effect on both the growth and dissolution processes of the lysozyme crystals. A decrease in the diffusion coefficient caused by the magnetic field might be one possible reason. But how does the magnetic field decrease the diffusivity?

To solve this problem, it would be useful to think about the initial stage of protein crystal growth. Many researchers have found that small aggregates appear in a supersaturated protein solution (Behlke & Knespel, 1996; Boué *et al.*, 1998; Minezaki *et al.*, 1996; Pusey, 1991; Wilson *et al.*, 1996). Tanaka *et al.* (1999) pointed out that small aggregates of protein molecules form in the initial 2–3 h after the solution preparation. Larger aggregates will appear later depending on the solution conditions. In our experimental conditions, the temperature program allowed aggregates to form, particularly in the lower part of the cell (because of the lower temperature). This was strongly supported by our concentration measurement. As seen from points m1–m3 in Fig. 2(*d*), the concentration in the lower parts of the cell increased first before the concentration started to decrease at around 25 h (*i.e.* the onset of rapid crystal growth). Conversely, the concentration in the upper part of the cell (Fig. 2*d*, point 4) decreased monotonically. Fig. 3 shows more clearly the changes in the lysozyme concentration at points 1 and 4. In our measurements, the largest concentration difference between points 1 and 4 was about 7.37 mg ml^{-1} at 21 h after starting the experiment. From the experimental results, we think that aggregates, probably in an amorphous state, form first then sink downwards owing to the gravitational force, resulting in a redistribution of the apparent concentration in the cell.

The discovery of the concentration redistribution is also in good agreement with previous work in the literature. Concentration gradients along the gravitational field in a supersaturated solution have been reported by Mullin & Leci (1969) and studied extensively in more recent years (Myerson & Izmailov, 1993; Izmailov & Myerson, 1996; Ohgaki *et al.*, 1991). In these investigations, the density gradient appearing in an aged solution was studied by measuring the density or concentration of solutions sampled from the container using a syringe (Mullin & Leci, 1969; Larson & Garside, 1986; Myerson & Lo, 1991; Ohgaki *et al.*, 1991). It was found that the density gradient appearing in an isothermal column was attributable to cluster formation over a sufficiently long period of time. In the present study, such density (or concentration) gradients can be supported directly by means of the *in situ* measurement of the concentration using interferometry, which is an accurate and non-invasive means compared with the sampling method.

As Myerson and coworkers pointed out (Sorell & Myerson, 1982; Chang & Myerson, 1986), the diffusion coefficient of the solute molecules decreases sharply with increasing concentration in the supersaturated solution. In our experiment, if the magnetic field changed the concentration redistribution, it might be easier to understand our findings. For instance, if the concentration gradient is larger under a magnetic field, it would strongly support the diffusion coefficient of protein in the solution being decreased in a magnetic field. However, to date we do not have any clear evidence to show that the magnetic field has changed the concentration redistribution. Based on the following reasons, we are inclined to believe that the magnetic field does not change the concentration redistribution. The movement of the species in the lysozyme solution in a magnetic field is affected by the following factors: (i) gravitational force, (ii) Lorentz force, (iii) Kelvin force, (iv) buoyancy force and (v) Brownian motion. Under the present experimental conditions, only the Lorentz and Kelvin forces are correlated with the magnetic field. Because the lysozyme solution is an almost non-conducting solution, the Lorentz force is negligible; as the magnetic field is highly homogeneous, the Kelvin force is also negligible. Therefore, we have to find other reasons for the experimental results.

It is expected that nucleation and crystal growth might become easier inside protein-rich aggregates than in the less dense surrounding solution, because in such cases they consume less energy. Haas & Drenth (2000) studied the interface between a protein crystal and aqueous solution. In their study, they described homogeneous nucleation and growth directly from the dilute but supersaturated solution: the protein molecules must first move to the surface of the crystal (or the crystalline nucleus), then stick to the crystal (or nucleus) surface with proper orientation because of the large anisotropy of the interaction between the protein molecules. In such a case the sticking probability is small. Furthermore, the molecules that do stick to the surface must diffuse to the growth sites to be incorporated into the crystal, resulting in a limited growth rate. In the case of growth from aggregates, the crystal or nucleus could be covered by a protein-rich film in which the protein molecules are quite mobile and have adequate time to adjust their orientation to be incorporated

into the crystal. Moreover, the sticking process from the bulk solution also becomes easier, as no orientation is necessary for molecules to be incorporated into the protein-rich film.

If aggregates formed in our experiments, as indicated from the above concentration measurement, nucleation and crystal growth inside the aggregates might preferentially occur. We assume that more crystals will be present in, or arise out of, the aggregates than in the bulk solution.

When these crystals are not large enough (typically smaller than a few micrometres; Ataka *et al.*, 1997), they will be suspended in the solution. As we know, the magnetic field will always exert a torque on lysozyme crystals because they are magnetically anisotropic. When the torque overcomes the thermal energy, the crystals will tend to be oriented along the magnetic field direction. Yamagishi *et al.* (1990) made a theoretical approach to the magnetic orientation of polymerizing fibrinogen and showed that 80% of the fibrinogen will be magnetically orientated when the following condition exists:

$$\Delta\chi H^2 = 15k_BT, \tag{2}$$

where $\Delta\chi$ is the anisotropy of the magnetic susceptibility of a crystal, H is the magnetic field strength, k_B is the Boltzmann constant (1.38×10^{-23} J K^{-1}) and T is the temperature.

In the case of lysozyme crystal orientation, the following equation is necessary for the orientation (Wakayama, 1998):

$$N_H = 120k_BT/\Delta KH^2, \tag{3}$$

where ΔK is the anisotropy of the magnetic susceptibility per unit cell (for lysozyme crystal $\Delta K = 5 \times 10^{-27}$ J T^{-2}) and N_H is the number of the molecules in a crystal necessary for orientation. In the present case, the magnetic field $H = 6$ T; therefore, the number $N_H = 2.7 \times 10^6$, corresponding to a size of about 0.37 μm. If many small suspended crystals of such a size or larger are oriented along one direction, we can imagine that a fraction of the solution has ordered structure. The orientation of the ordered solution fraction should be relatively stable if the magnetic field is maintained. Such an ordered structure might contribute to the decrease in the diffusion coefficient and the increase in viscosity and even make the solution exhibit a birefringence property, as recently discovered by Zhong *et al.* (2001). The above idea can be proposed as a suspended crystal model for explaining the phenomena concerning the effects of magnetic field on protein crystal growth and dissolution. The model is schematically illustrated in Fig. 4.

Fig. 4(*a*) is an aggregation domain with a small crystal inside. The solution was proposed to contain such domains and aggregates without any crystals, as illustrated in Fig. 4(*b*). In Fig. 4(*b*), there is no magnetic field applied and thus the crystals are randomly distributed without any orientation. When a strong magnetic field is applied, crystals with adequate size will be oriented in one direction, as illustrated in Fig. 4(*c*). Now let us consider the diffusion of a protein molecule at the two cases with and without magnetic field. When there is no torque exerted on the crystals, the molecule can diffuse by rotating the domains or crystals in the solution if the latter block the way. When there is a torque exerted on the crystals, the molecule or small domain has to diffuse by pushing the oriented domains or crystals (see Fig. 4*c*) if they block the way, or by changing the migration direction, which will result in a longer diffusing distance compared with the case without a magnetic field. Apparently, oriented suspended crystals act as stronger barriers to diffusing molecules than non-oriented crystals. This might be the reason for the decrease in the diffusion coefficient of molecules in the magnetic field.

5. Conclusions

In this paper, we have conducted an investigation into magnetic field effects on the dissolution process of tetragonal lysozyme crystals. The concentration distribution was measured in both the growth and dissolution processes by Mach–Zehnder interferometry. Using a steady and homogeneous magnetic field of 6 T, the following conclusions were obtained.

(i) The magnetic field can damp the dissolution process of lysozyme crystals.

(ii) The diffusion coefficient of lysozyme molecules in the solution was decreased in a magnetic field compared with the case with no magnetic field.

(iii) Under a vertical temperature gradient, the lysozyme solution undergoes a redistribution of concentration before the onset of crystal growth in the absence of a magnetic field. The concentration in the lower (colder) supersaturated part of the cell increased, while the concentration decreased in the upper (hotter) undersaturated part. This phenomenon, similar to that previously observed in an aged supersaturated solution under isothermal conditions, was attributed to the formation of aggregates.

(iv) A model of suspended crystals was proposed to explain the magnetic damping of lysozyme crystal-growth and dissolution processes.

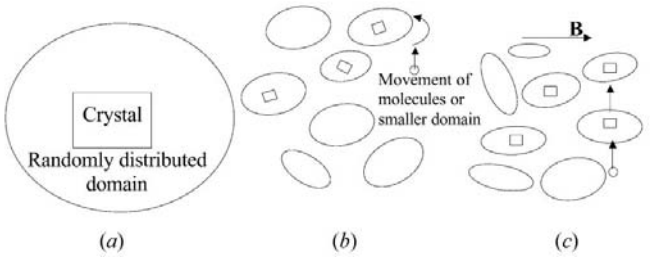

Figure 4
A suspended crystal model to explain the diffusivity decrease of lysozyme molecules in a magnetic field. (*a*) A single domain (or aggregate) with a crystal inside. The solution a period of time after preparation is postulated to be composed of such domains (dense solution) with and without crystals inside and less dense surrounding solution. (*b*) Solution without magnetic field. The suspended crystals (in the domains) are distributed randomly without an orientation. Small domains or molecules may diffuse by rotating the domains if they block the pathway. (*c*) Solution in a strong magnetic field. Small domains or molecules may diffuse by pushing the domains if they block the pathway, or by changing the migration direction, thus extending the diffusion distance.

The discovery of the decrease in diffusion coefficient, together with the discovery of viscosity change and the possible ordered 'solution structure' or ordered suspending crystals under magnetic field, might open the way to a fundamental understanding of the physics of magnetic field interaction with the protein solution and the growth process of protein crystals.

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