Effects of a magnetic field and magnetization force on protein crystal growth. Why does a magnet improve the quality of some crystals?

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Probable reasons why some protein crystals grown in a magnet exhibited better quality than control are discussed as follows. (1) Sedimenting three-dimensional nuclei are able to have the same orientation as the underlying, mother crystal into which the nuclei merge. (2) Protein solution may become more viscous, leading to reduction of convection. (3) If an upward force is generated by use of an inhomogeneous magnetic field, the effects of the density differences can be made less significant, causing the reduction of natural convection and the retardation of crystal sedimentation.

1. Introduction

The quality of single protein crystals is critically important to carry out detailed structure analyses of protein molecules by X-ray crystallography. Accurate and reliable structures are available when X-rays diffract to higher resolution (higher Bragg angles) in all directions. Therefore, a number of a more or less general means to improve the quality of crystals in the course of crystal growth are being developed.

Applying a magnetic field or magnetization force has recently been shown as a potential means for this purpose. Magnetization force F_M results from spatial inhomogeneity of the magnetic induction B:

$$F_M = \chi B (\text{grad } B), \qquad (1)$$

where χ is the magnetic susceptibility. All substances are magnetized in a magnetic field, and either gain or lose free energy. Therefore, by making use of a gradient of B (i. e. grad B), we can induce a force to minimize the free energy. Diamagnetic substances (including a majority of proteins and water) have a negative χ value, and try to get rid of the magnetic field. The absolute values of χ are extremely small compared with ferromagnetic materials, and therefore the magnetization force for a diamagnetic material has been neglected both experimentally and theoretically in most cases. However, owing to the recent developments in superconducting materials and superconducting magnets, generation of a high B as well as a high B (grad B) value has increasingly become feasible without a liquid helium refill or the high cost of flowing electric currents through a solenoid. This means that we can use a strong magnetic field and a strong magnetization force in normal laboratories.

Lin *et al*. (2000) grew snake muscle fructose 1,6-bisphosphatase crystals of hexagonal symmetry. They found that the crystals that grew in the presence of both a magnetic field and an upward magnetization force were reproducibly the best in quality as judged from the resolution. In this case, the resolution was defined as the highest resolution shell with a S/N ratio ≥ 2 in collected data. At the center of the magnet, where a homogeneous magnetic field of 8 - 10 T existed but no magnetization force ((grad B) = 0), the crystal quality seemed to be better than the control obtained without a field or a force. When both a downward force and a magnetic field were present, the crystal quality was the worst. Sato *et al*. (2000) grew orthorhombic hen egg-white lysozyme crystals in a homogeneous magnetic field of 10 T, and found that the widths of the rocking curves became consistently narrower than the crystals grown in its absence. Sazaki *et al*. (2002) compared oriented BphC crystals grown in a magnet with ones that grew in a magnet but that did not exhibit magnetic orientation. The crystals that were oriented in a magnetic field were reported to be of better quality than those without orientation.

In this paper, we present three reasons (sections 2.1, 2.2, 2.3) that we consider to be most probable why a magnetic field and a magnetization force show the potential ability to improve the quality of protein crystals. The papers we have so far published (Ataka *et al*., 1997; Wakayama *et al*., 1997; Sakurazawa *et al*., 1999; Lin *et al*., 2000; Zhong & Wakayama, 2001; Wang *et al*., 2002) aimed primarily at reporting various experimental facts that take place in a magnet. The purpose of this paper is to report and discuss the reasons, based on our present understanding, why favorable changes occur in a magnet, especially on the quality of some protein crystals.

2. The merits of using a magnet

2.1. Effects of magnetic orientation on 3-D nucleation

2.1.1. Sedimenting crystals. Hen egg-white lysozyme crystals of tetragonal symmetry are known to orient when grown under a magnetic field (Sazaki *et al*., 1997). We used this phenomenon to know the initial stage of crystallization (Ataka *et al*., 1997). A magnetic field of 1.6 T was sufficient to orient almost all the crystals if applied over the whole period of crystallization. In the case of α-amylase and BPTI, it was shown that even a magnetic field of 1.25 T was sufficient (Astier *et al*., 1998). We applied a field of 1.6 T only for the initial 2 or 8 h after supersaturating the protein solution by adding a crystallizing agent, NaCl. The solution was taken out of the magnet after this period. At this point, crystals were hardly visible in the solution. However, at 24 h after supersaturation, we could clearly observe under a microscope a number of large enough tetragonal lysozyme crystals. The ratio of the oriented crystals to the total number was 25% when we removed the solution from the magnet at t = 2 h, and 68% when t = 8 h. Moreover, we could observe that almost all the oriented crystals were larger than the unoriented ones. All the crystals, oriented and unoriented, were stuck to the bottom glass surface, probably due to the same, considerably strong adhesion force as discussed by Tsekova *et al*. (1999). These results could well be interpreted if we considered that 25% or 68% crystals were already growing at the bottom of the vessel (after sedimentation) when the magnetic field was turned off. The crystals continued to grow afterwards with the same magnetically induced orientation they had acquired. On the other hand, the unoriented crystals were considered either to start growing after the magnetic field was switched off or to have lost the magnetic orientation. The consistently smaller dimension of the unoriented crystals suggested that they started growing later than the oriented ones. Thus by using the magnetic orientation we could flag each crystal which was already present at the bottom of the vessel at t = 2 or 8 h. These crystals were fed with protein molecules for additional tens of hours, until they reached an easily observable size, and photographed for orientation observation. From among a large number of crystals we could pinpoint the ones that existed at a particular time by turning off the magnetic field at that time and observing their orientation.

As an alternative, we started to apply the magnetic field 2 or 8 h after supersaturating the solution. In this experiment, we could observe that the oriented crystals were consistently smaller than the unoriented ones. The ratio of the oriented to the total number of crystals was 46% and 14%, when the magnetic field was turned on 2

and 8 h after supersaturating the solution, respectively. These facts show that the magnetic field of 1.6 T does not have the ability to rotate the crystals that already adhere to the vessel. In other words, once a crystal adheres to the vessel, it is impossible to reorient it magnetically, owing to a stronger adhesion force. Obviously a crystal must be suspended in the bulk solution to be able to acquire magnetic orientation. Since most of the crystals were oriented, they must have started to grow within the solution and orient while they were floating, then sedimented to the bottom, where the adhesion force additionally operated. Furthermore, from the determination of the smallest magnetic field strength needed to orient almost all the crystals, we also concluded that they started to sediment when their size reached a few μm (Ataka *et al*., 1997). Astier et al. (1998) gave another more direct evidence that under a microscope most crystals start to grow in the bulk of the solution. It is added that these conclusions have important implications for microgravity experiments, since the magnitude of gravity can exert a profound influence on protein crystal growth through the presence or absence of sedimentation. This is an evident effect of gravity that is different from the more often discussed presence or absence of convection.

2.1.2. Three-dimensional (3-D) nucleation. Before we carried out the above experiments, it had been demonstrated that 3-D nucleation is a common crystal growth mechanism for biological macromolecules (Malkin *et al*., 1995, 1996). This mainly came from atomic force microscope (AFM) observations of the surfaces of a number of protein and virus crystals. The 3-D nucleation is the attachment of tiny crystals of a size in the range of μm from the bulk solution onto the existing crystal surface. Eventually all of them merge into one. The 3-D nucleation is not common for the crystal growth of inorganic materials, but is frequently observed for biological macromolecules (Malkin *et al*., 1995, 1996). They do not necessarily consider that the tiny crystals sediment; they may be transferred by a solutal flow including convection. However, since AFM usually observes the horizontal surface of a crystal from above, we consider that the 3-D nuclei can be the successively nucleating and sedimenting crystals, which we considered in 2.1.1. An important observation of Malkin *et al*. (1995, 1996) is that the eventually obtained large crystal, used for structure determination, can contain a number of crystals that originated in the bulk solution independently and have merged.

Recently, Astier *et al*. (2001) also reported a similar AFM observation on α-amylase crystals. They demonstrated that a microcrystal of around 1 μm in size is incorporated into the larger one under observation, producing a macrodefect. They further discuss that this incorporation could be the reason for poor crystal quality in X-ray diffraction.

In 2.1.1 we concluded that, in the presence of a magnetic field, the nuclei sediment to the bottom after having acquired a magnetic orientation. By making use of this ability of the magnetic field, we can give the sedimenting and merging 3-D nuclei the same orientation as the existing mother crystal (Lin *et al*., 2000). In the case of 2.1.1, a magnetic field of 1.6 T was sufficient to orient almost all the crystals. However, if the field is stronger, more of the smaller crystals can acquire orientation in the course of crystal growth. On the other hand, the existence of a magnetic field does not affect the crystal size at which sedimentation starts (apart from a possible, small influence coming from orientation). Therefore, we consider that the application of a field of about 10 T by a superconducting magnet in the whole course of the crystal growth may be efficient to improve the crystal quality, since we can align the 3-D nuclei with the same orientation as the mother crystal with which they merge. The fact that the magnetic orientation is expected for all the crystal symmetries except for cubic has been discussed by Sakurazawa *et al*. (1999).

2.2. Viscocity increase in a magnetic field

Recently, Zhong & Wakayama (2001) showed that the viscocity of supersaturated lysozyme solutions, measured by the falling sphere method, increased in the presence of a magnetic field of 10 T. At present, the mechanism of the magnetic increase of viscosity on the molecular level is unclear. However, if we consider that many crystals are suspended in the bulk solution at the initial stage of growth (2.1.1), the viscosity increase may to some extent come from their magnetic orientation. A magnetic increase of viscosity is also reported in human blood, and has been explained by the orientation of red blood cells (Haik *et al*., 2001).

At the same time, we have long been interested in the changes that occur gradually in a supersaturated lysozyme solution (Tanaka *et a*l, 1996, 1999; Michinomae *et al*., 1999). We have shown that, before the crystals start growing from their nuclei, another process in which a majority of lysozyme molecules participate proceeds in supersaturated solutions. This process could be related to the liquid-liquid phase separation, shown recently to have an important relationship with the crystal growth (Drenth & Haas, 1998; Haas & Drenth, 1999). The viscosity increase in a magnetic field may in part be related to a change in this process.

If the viscosity increases in a magnetic field, then convection should be effectively reduced. The quality improvement of protein crystals in a magnetic field could arise from reduced convection due to the viscosity increase. Of course, we can increase viscosity of a protein solution by a number of other means including addition of PEG or a gel. Our intention here is not to propose the use of a magnetic field as a means to increase viscosity, but is to enumerate probable reasons why a magnet can improve the quality of protein crystals.

Yin *et al*. (2001) showed that the presence of a magnetic field reduces both crystal growth and crystal dissolution rates using a layer of tetragonal lysozyme. Elucidating the reason was considered to be a challenge. The observations of Yin *et al*. can be explained if we consider that the viscosity increase under a magnetic field brought about the reduction of growth and dissolution rates.

The two reasons discussed in 2.1 and 2.2 are effective, both in a homogeneous and an inhomogeneous magnetic field. The reason discussed in 2.3 concerns the magnetization force that is generated only in an inhomogeneous magnetic field.

2.3. Reduction of the effects of density differences in the presence of an upward magnetization force

By deliberately situating the sample solution for crystallization at a proper position, where the B (grad B) product becomes large in a vertical superconducting magnet, we can apply an upward magnetization force on protein solutions and crystals (Wakayama *et al*., 2001). Numerical simulation has shown that the presence of this force can reduce convection (Qi *et al*., 2001). In the simulation, hydrodynamic equations were solved in the presence of a magnetic field and magnetization force. Realistic values of kinematic viscosity, density, and electric conductivity of crystallizing aqueous lysozyme solution were used. Depletion of protein solutes around a growing crystal occurs, especially when the transport is a limiting factor of crystal growth. A decrease in density in the vicinity of a crystal surface by 1% from the bulk was considered to be the driving force of convection. Our simulation results show that an upward magnetization force can effectively reduce the convective flow.

A simulation also showed that the Lorentz force only (without magnetization force) has negligible effects in reducing the convection Damping by the Lorentz force is induced when an electric current traverses a magnetic field even when the field is homogeneous. The electric conductivity used in our simulation was that of a 3% NaCl solution ($4.13\ \Omega^{-1}\ m^{-1}$). When we add protein to this salt solution, the

electric conductivity should become still lower. On the other hand, the electric conductivity of semiconductor melts, for which magnetic damping of convection is already industrially used, is higher by 5 - 6 orders of magnitude. For molten Si, the electric conductivity was considered to be 1.25 $M\Omega^{-1}$ m^{-1} (Oshima *et a*l., 1994). Therefore, reduction of convection by Lorentz force, effective for metal and semiconductor melts, does not work for aqueous solution of proteins. Instead, we have to rely on magnetization force that applies even on electrically insulating or low-conducting materials. In this case, however, the direction of the magnetization force had to be upward for efficient reduction of convection.

When an upward magnetization force exists, the sedimentation that is discussed in 2.1.1 is also retarded, since the driving force of sedimentation is the density difference between the crystal and solution. If the density difference becomes less significant in the presence of an upward magnetization force, the suspended crystals can keep their suspended state for a longer time. This could be an additional benefit from the magnetization force.

3. Conclusions

It is widely accepted in the field of structure determination of proteins by nuclear magnetic resonance (NMR) that the presence of a strong magnetic field of 10 – 15 T does not have harmful or artificial effects on the determined structure. The magnetic energy is usually too small for an individual protein molecule to be affected. However, in the course of crystal growth, 10^{12}-10^{18} protein molecules assemble in a regular array. Superposition of extremely small magnetic anisotropic energy during the crystal growth eventually overcomes thermal energy; the result is the magnetic orientation phenomenon (Ataka *et al*., 1997; Sakurazawa *et al*., 1999). Given the obvious technological developments over the last decade which have enabled NMR spectroscopy to be widely introduced into structural genomics and proteomics, we thought of using a magnet for improving the quality of protein crystals for X-ray crystallography.

Coming back to the examples given in the Introduction, quality improvements of some protein crystals that occurred in a magnet. Lin *et al*. (2000) could have used all the advantages discussed in 2.1, 2.2 and 2.3. The results of Sato *et al*. (2000) could have used the merits except for the third one, since the magnetic field used was homogeneous. The results by Sazaki *et al*. (2002) can also be understood, if we consider that the crystals that did not orient in the magnetic field could not benefit from the first merit, whereas the oriented ones could receive full benefits. We thus consider that the use of a magnet could further be tried as a means to improve the quality of protein crystals. Post-growth treatments (e.g. cryo-cooling and annealing) in a magnet are also an interesting possibility.

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