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(Review)

Do We Need Microgravity to Improve the Diffraction Properties of Protein Crystals?

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Abstract

This paper summarizes the panel discussion on the impact of microgravity on the diffraction properties of protein crystals. Convection-free environments have been considered as ideal environments for the growth of well diffracting protein crystals. Hence, since 1981 protein crystallization experiments have been performed in microgravity with varying degrees of success. During the discussion we addressed the effects of the crystal growth environment on crystal quality and perfection and we discussed whether higher crystal perfection leads to better X-ray diffractivity.

Keyword(s): Protein crystallization, Depletion zone, X-ray quality, Microgravity
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1. Introduction

The goal of structural biology is to understand biological processes at the atomic/molecular level. In this research field one of the central tools is X-ray diffraction. As the quality of the macromolecular structure is directly related to the quality of the X-ray data (**Fig.1**) obtaining well diffracting crystals is crucial. Crystals grown in a diffusive environment have been believed to be of superior quality for a wide variety of macromolecules. Hence, numerous protein crystallization experiments have been performed in microgravity with varying degrees of success[2,3]. As the number of microgravity experiments is limited, thorough statistical comparisons of X-ray datasets collected from space grown crystals with those from earth grown crystals are scarce[1,4].

In parallel scientific studies on protein crystal nucleation and growth have been performed with the aim to identify the parameters that influence crystal quality and perfection. To obtain highly ordered crystals two main requirements should be fulfilled: (I) proper orientation of molecules and (II) reduced incorporation of impurities. Impurities induce microdefects leading to local lattice strain.

The first factor is directly correlated with the growth rate of the crystals, and as such by the growth mechanism (e.g. spiral growth and 2D nucleation) of the crystal and the distribution of the supersaturation at the crystal surface. The faster solute molecules will incorporate into the lattice, the less time molecules will have to acquire the appropriate orientation. In the case of protein molecules this orientational entropy seems to be very important due to the high complexity of the molecules and their large size, both considerably increasing the time necessary for proper orientation. Although this general idea was confirmed at a wide range of supersaturations[5] it is not clear if it is generally applicable because the supersaturation at which the lowest quality crystal (in terms of resolution) were obtained was unrealistically high. But not only the level of supersaturation is important and also the distribution of the supersaturation at the crystal surface, which is not uniform, plays a crucial role. A convective regime results in an inhomogeneous distribution of supersaturation due to convection plumes at the

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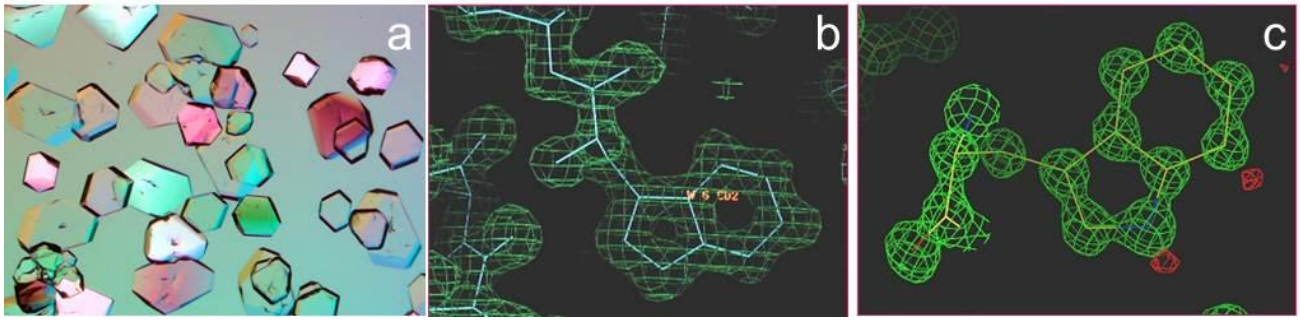

Fig. 1 Hen-egg tetragonal crystals observed by optical microscopy (a) and X-ray density maps at 1.12 (b) and 0.89Å (c) resolution. The lateral scale in Fig.1 is approximately 2 mm.

surface of the growing crystal, causing the development of defects leading to a hopper crystal[6]. 3D laser interferometry measurements confirmed that the inhomogeneity of surface supersaturation has a negative impact on crystal perfection and diffraction quality[7]. In contrast a diffusive regime assures a more homogeneous supersaturation distribution resulting in a more stable crystal morphology[8].

The second factor is directly related to the amount of impurities incorporated and the lattice disorder they provoke. The most effective way of avoiding the detrimental effect of impurities on the quality of diffraction data is by eliminating them from the growth solution. But, in the case of protein solutions this is by no means trivial, and we should assume that most protein preparations contain at least 5% of impurities. Moreover, protein molecules tend to denature during growth[9]. Once impurities are in the growth solution, different approaches exist to reduce the impurity effect, depending on the characteristics of the impuritymolecules. In the case of impurities that are preferentially incorporated into the crystal lattice, their incorporation can be significantly reduced when an impurity depletion zone is formed. This model, introduced by Chernov[10], argues that, upon the start of crystal growth, impurities in the proximity of the crystal will be incorporated and hence, the surrounding solution will be cleansed from impurities. Consequently, at later times, growth will take place from a more pure solution and fewer impurities will be incorporated. Recent experimental data do not support this simple idea that was predicted to be valid in cases where the partition coefficient is smaller than unity[11]. To fully understand this problem, more experimental data is needed.

On the whole it is clear that the presence of a solute/protein depletion zone (PDZ) and an impurity depletion zone (IDZ) are not a prerequisite to grow high quality crystals. For a better understanding of the relation between growth environment and crystal quality and diffractivity, several studies were performed on the one hand in convection free environments such as crystal growth in gel[12], the ceiling method[8] and crystal growth in magnets[13,14] and on the other hand in forced convection[15]. X-ray topography studies were initiated to elucidate the relation between crystal perfection and the growth methods/conditions[16].

During the panel discussion we addressed different environments for crystal growth and their effect on impurity uptake. We discussed the internal organization of a well diffracting crystal. We addressed the question as to whether the highest quality crystal results in the best X-ray dataset. The experiences learned from crystallization experiments in space during JAXA-PCG missions were presented. The utilization of microgravity conditions for the growth of better quality crystals was discussed.

2. Crystallization in Different Regimes

2.1 Crystallization in Convection-Free Environments

2.1.1 The Protein and Impurity Depletion Zone

Figure 2 shows the depletion of proteins and impurities in the vicinity of the growing crystal surface. In convection-free environments, the degree of the depletion will be enhanced and an impurity and protein depletion zone will be established. These depletion zones affect the impurity incorporation into the crystal. The degree of the impurity incorporation is usually characterized by the partition coefficient. We can consider two different definitions as follows:

$$K = \frac{C_{ic}/C_{pc}}{C_{il}^{s}/C_{pl}^{s}} \qquad and \qquad K' = \frac{C_{ic}/C_{pc}}{C_{il}^{\infty}/C_{pl}^{\infty}} \qquad (1)$$

C denotes the concentration; the subscripts “p”, “i”, “l”, and “c” indicate protein, impurity, solution (liquid), and crystal, respectively; the superscripts “s” and “∞” indicate at surface and at bulk, respectively. K is usually termed the equilibrium partition

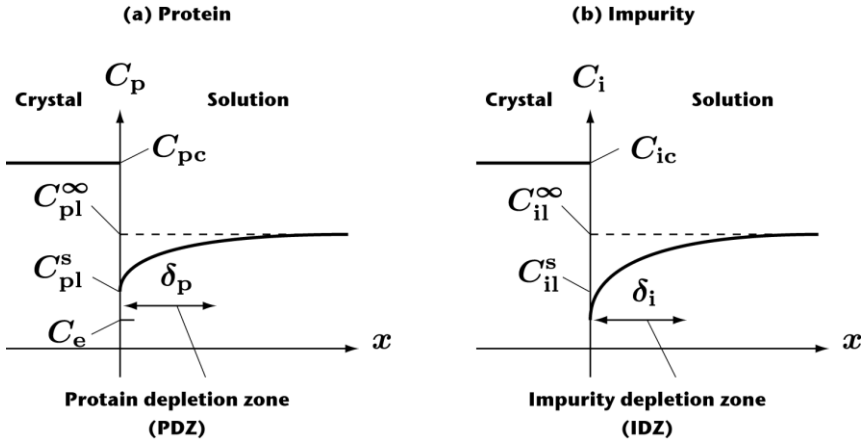

Fig. 2 Concentration profiles *C(x)* of protein (a) and impurity (b) in non-convective solution as a function of the distance from the growing crystal surface, *x*. The subscripts "p", "i", "l", and "c" indicate of protein, of impurity, in solution (liquid), and in crystal, respectively. The superscripts "s" and "∞" indicate at surface and at bulk, respectively. Protein and impurity are depleted at the vicinity of the crystal surface because of their incorporations into the growing crystal. δ is the typical width of the depletion zone. C_e is the solubility of protein.

coefficient and is determined by the local properties at the crystal-liquid interface; as such, *K* is a quantity that is not affected by the development of the depletion zones. On the other hand *K'*, the effective partition coefficient varies with the development of the depletion zones. When $K' > K$ the depletion zones will promote incorporation of impurities into the crystal and vice versa. Note that $K' \approx K$ when both depletions are negligible. We derived the relationship between C^s and C^∞ as a function of the bulk supersaturation σ^∞ based on the analysis of Chernov[10] to obtain

$$\frac{K'}{K} = \left[1 + \frac{(K\nu - 1)\alpha\sigma^\infty}{(1+\alpha)(1+\sigma^\infty)}\right]^{-1} \tag{2}$$

where $\alpha = p\beta_{st}\delta_p/D_p$ is a non-dimensional parameter (see below), $\nu = \frac{\delta_i D_p}{\delta_p D_i}$ denotes the ratio of diffusivities of protein and impurity, δ is the typical width of the depletion zone, *p* is the slope of the vicinal surface, β_{st} is the step kinetic coefficient, and *D* is the diffusivity. The crystal growth rate is limited by the diffusive transport when $\alpha \gg 1$ and by the incorporation kinetics at the crystal surface when $\alpha \ll 1$. Eq. (2) suggests that $K\nu$ is the parameter that determines whether the impurity incorporation is promoted ($K\nu < 1$) or inhibited ($K\nu > 1$) by the development of the depletion zones. The parameters α and/or σ^∞ just determine the degree of the enhancement or inhibition.

When $K < 1$, the poor impurity incorporation hinders the development of the impurity depletion zone, so the relative impurity concentration, C_{il}^s/C_{pl}^s, will be increased. This may enhance the impurity incorporation into the crystal. However, Eq. (2) shows that in cases where $K < 1$ the impurity incorporation will be suppressed if $\nu \gg 1$, e.g., large impurities having a small D_i.

2.1.2 Crystallization in Gel

A convective-free growth environment can be obtained on Earth in gelled solution. Hence, the question arises: which method is the better choice for obtaining crystals for structural determination? The answer to this question, in a nutshell, is the following: from the view point of structural resolution, there should be no obvious differences between crystals grown from a mass diffusive environment created by a gelled solution on Earth and microgravity in space. *In situ* observation of protein crystal growth in gelled solution showed that agarose gels with a concentration as low as 0.025% can induce diffusive impurity filtering[17]. In addition crystals grown in gel on ground and in solution in microgravity did not show any significant difference in diffraction properties[4]. Hence, both environments produce crystals of equal quality. Taking into account that gelled solution experiments are much less time consuming and several orders more cost-effective than protein crystallization in microgravity, it seems reasonable to postulate that gelled solution should be our first choice for improving the diffraction quality of biological macromolecules. This choice is further motivated by the large number of macromolecules reported to produce "good" crystals in agarose gels[12]. But, it goes without saying that, if the gelling agent has a negative influence on our protein molecules, or their crystallization behavior, then microgravity should be considered as a valid option.

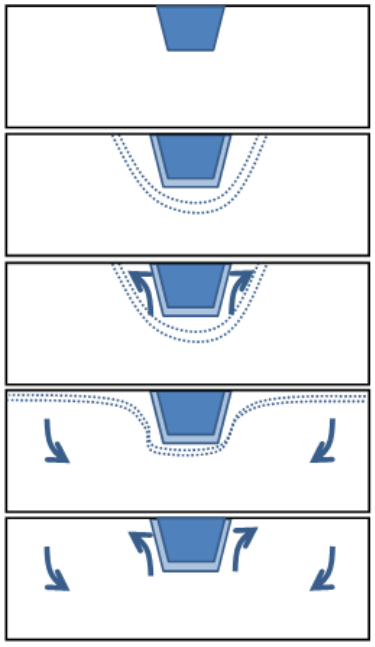

Fig. 3 Schematic illustration of the formation of convective flows around a growing crystal at a ceiling position in a flat cell. Dotted lines indicate the concentration distribution around the crystal. The differences in density in the solution induce the convection flows around the growing crystal when the sample cell is flat. On the contrary in deep sample cells the solution at the top will be quasi stagnant (cf. the 2 top images).

2.1.3 Crystallization using the Ceiling Method

In the ceiling method a ground-based convection-free environment for crystal growth is created at the top of the growth cell[8]. Crystals growing at the bottom of a container induce convective flows on the top of their growing surface: during growth the protein concentration around the crystals decreases, the density of the solution around the crystal decreases as compared to the rest of the container resulting in an upwards flows. In contrast, when crystals grow from the top wall of the container, the solution around the crystals is expected to be stagnant, because the part of the solution with smaller density is located at the upper part of the container where it is stable. Using this set-up effective partition coefficients K' of several impurities were measured for lysozyme crystals. As expected from the depletion zone theory crystals grown at the ceiling position impurity uptake was decreased when $K' > 1$ and increased when $K' < 1$. However, the authors claimed that even when $K' < 1$ the crystals grown by the ceiling method resulted in higher resolution X-ray data sets because of the slower growth rate. It is clear that the sample cell needs a minimum height to reduce convective flows (**Fig.3**)

2.1.4 Crystal Growth in Forced Convection

At Osaka University within the SOSHO project, crystallization of various proteins was achieved by introducing solution flow. For 70% of the tested proteins large high-quality crystals were obtained and their 3D structures could be determined[15]. In order to clarify the effect of controlled solution flow on the quality of the X-ray data sets, the relation between solution flow and the perfection of the protein crystals was examined. In some cases the improvement in X-ray diffraction quality was a consequence of the better morphology and/or the larger size of the crystals obtained in solution flow. This might be explained by the uniformization of the growth condition around the growing crystals[18-21], (**Fig.4**). However, in other cases no apparent changes were observed in crystal morphology and/or crystal size, notwithstanding a dramatic improvement in X-ray diffraction quality. A detailed observation of the crystal surface provided information on the relationship between crystal defects and solution flow. Surprisingly, the presence of dislocations, which are one of line defects, tended to increase by increasing the solution flow rate. In contrast, the microdefects tended to decrease by solution flow. As already reported in Ref.22, dislocations do not have a large impact onX-ray diffraction quality. During the growth process, protein crystals accumulate strain as a consequence of lattice disorder[10]. Strain aggregates along the introduced dislocations, and overall strain in a crystal possibly reduced, resulting in a higher X-ray diffraction quality. Moreover, dislocations are the source of spiral hillocks and consequently, when the dislocation density increases, the growth mechanism may shift from a 2D nucleation to a spiral growth mechanism[27]. The spiral growth mechanism reduces the impurity incorporation into the crystal[15,23,24] and as such also improves its X-ray diffraction quality.

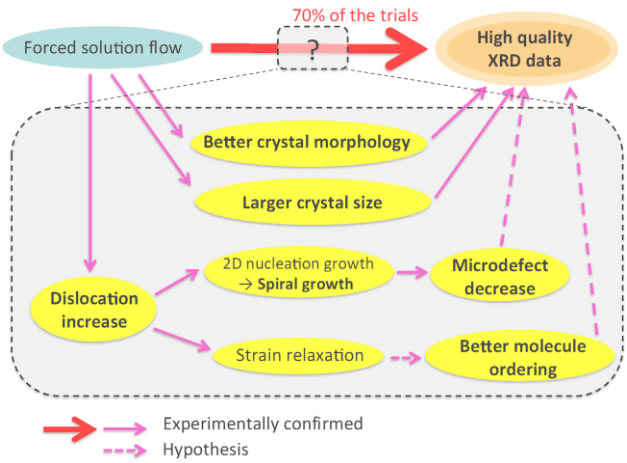

Fig. 4 Summary of the effects of controlled solution flow on protein crystal growth and XRD quality.

3. What are High-Quality Protein Crystals?

In general, it is said that a high percentage of high-order reflections (high resolution) need to be collected from high-quality crystals in order to obtain accurate 3D structures. The question arises: what are high-quality protein crystals that give rise to high resolution X-ray data sets? If the crystal quality degrades due to cracks, many high-order reflections cannot be detected: the integrated intensities of the diffracted wave become weak, as shown in **Fig. 5**. In contrast for a perfect crystal multiple diffraction occurs leading to the attenuation of the integrated intensities of the diffracted wave. Therefore, an ideal mosaic crystal is needed, which predominantly generate the integrated intensities of the diffracted wave by kinematical diffraction. Hence, the misorientation between the subgrains and the size of the subgrains of the crystal are crucial. Quite surprisingly the most perfect crystal doesn't provide the best X-ray data set.

By analyzing the full width at half-maximum (FWHM) values of X-ray rocking-curves, it was shown that imperfections in lysozyme crystals are predominantly caused by the misorientation of the subgrains[16]. For glucose isomerase crystals the misorientation of the subgrains was smaller by one order of magnitude and they were classified as perfect crystals. In fact, clear equal-thickness fringes, which are attributed to the Pendellösung effect, were observed in the region of a tapered glucose isomerase crystal with wedge-like edges, using synchrotron monochromatic-beam X-ray topography[25]. This indicates that multiple diffraction occurs in a glucose isomerase crystal. X-ray diffraction data sets are generally collected under cryogenic temperature. The misorientation between the subgrains might be increased under cryogenic temperature. As such the diffractivity of perfect crystals, such as those from glucose isomerase, could be enhanced by controlling the subgrain formation under cryogenic temperature.

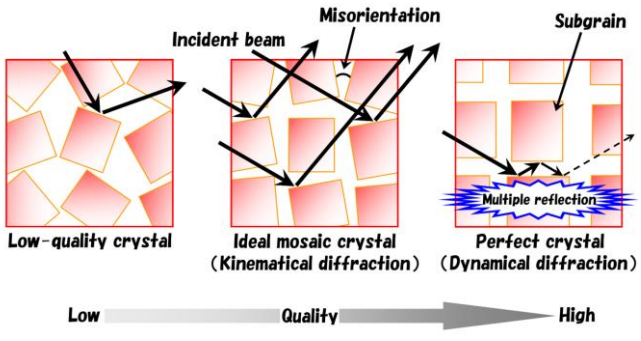

Fig. 5 Schematic of the integrated intensities of the diffracted waves from crystals of various qualities.

4. Experiences Gained from JAXA Protein Crystal Growth (PCG) Experiments in Microgravity

Protein crystallization experiments have been performed by JAXA for nearly 20 years. At present JAXA routinely launches protein samples about twice a year. Within the JAXA PCG project a lot of methods and devices, including crystallization cell, optimization methods, simulation programs, crystal harvest and cryo-protection techniques etc., were developed to fix a standard protocol for the handling of users' proteins.

The experiences gained from the PCG are the following: (1) microgravity improves the maximum resolution, Rmerge and mosaicity of the X-ray diffraction, and (2) microgravity reduces the growth of clustered crystals and increases the growth of single crystals[3]. Based on "the theory of the protein and impurity depletion zone" an easy method to estimate the parameter D/β was developed (D is the diffusion constant and β is the kinetic constant for the protein molecule). This parameter can be used as an index to estimate the effect of microgravity on crystal growth. It was found that a value of D/β less than 3mm results in auto-enhancement of the filtration effect around the growing crystal leading to better-quality crystals. That is the reason why one recommends users to use viscous precipitant to reduce D and further purification of the sample to increase β[26]. The JAXA PCG project does not recommend gelation because gels reduce β. Numerical models showed a major difference in impurity concentration between the center part and the surface of the crystal. This suggests that the X-ray quality is not uniform inside the crystal[3].

Measurements of the local diffraction pattern from lysozyme crystals by using micro-beam X-ray light source showed anisotropy in the unit cell dimensions, possibly caused by the impurity attachment. This finding suggests that most of the crystal is not a "perfect crystal". The extent of the non-uniformity in space-grown crystals is different from the one in crystals grown on Earth. There may be some best position to focus X-rays for the data collection.

5. Conclusive Remarks

In conclusion we summarize the opinions of the panelists.

Microgravity doesn't improve the diffraction properties of protein crystals in all cases. The microgravity effect depends on the protein and the impurities. One has to consider case by case.

In cases where impurity incorporation into protein crystals decreases the X-ray quality of the crystals, the answer to the question "Does microgravity improve the quality of protein crystals?" is YES.

Impurities in the protein solution are unknown, so you cannot always predict if microgravity will be beneficial.

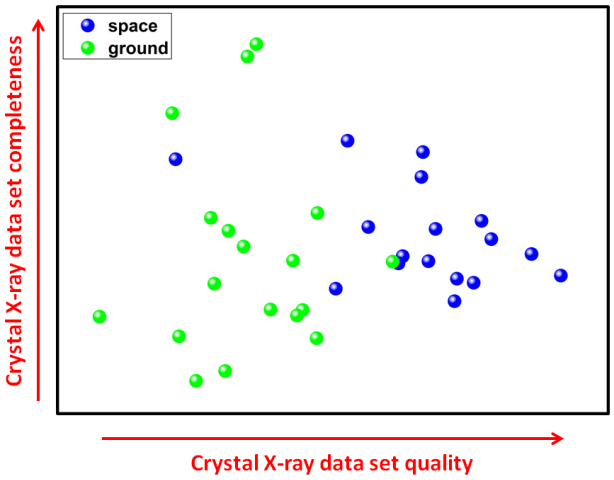

Fig. 6 Statistical quality analysis of 35 X-ray data sets of space and ground grown ferritin crystals (17 PromISS-4 "space" crystals and 18 from the ground control) as reported in Ref.1. Sixty-three parameters commonly used as indicative of X-ray data quality were analyzed. This highly dimensional "quality parameter dataset" was reduced using a principal component analysis. The differences between the two groups can be attributed to the first principal component and reflect the superior quality of the space crystals.

There is no convection-free environment on ground that is beneficial for all systems:

- for some systems the gel itself is an impurity
- the ceiling method is not always convection-free
- magnets can influence the growth kinetics

As crystallization in gels is cheap it is worthwhile to start by performing crystallization in gel environments.

In contrast to convection-free environments, crystal growth in forced flow environments might be beneficial for crystal quality too.

It should be noted that high-quality protein crystals are not the perfect crystals for X-ray structural analysis.

One good diffracting crystal is sufficient for structural analysis: the number of crystallization experiments in microgravity is limited. Although the probability of growing a good crystal on earth might be smaller, a crystal of similar quality can be obtained because the number of trials on earth is only limited by the amount of available protein.

In order to draw conclusions about the superiority of microgravity crystallization for X-ray diffraction a full statistical analysis needs to be performed on a large number of crystals (see **Fig. 6**)[11].

6. Future Perspective

We start to understand why microgravity conditions can be beneficial for the growth of small crystals. Due to the suppressed convection or flow, the incorporation of impurities (self-purification) is decreased and the crystal morphological stability is improved.

However, for larger crystal the situation is much more complex. For different growth mechanisms and degrees of impurity incorporation, the positive or negative effect of microgravity will be influenced by at least three factors: the degree of supersaturation, the homogeneity of the distribution of the supersaturation at the crystal surface and the flow speed of the solution. If we want to apply the currently available growth models and theories of inorganic crystals to the growth of protein crystals, we need to precisely measure, in microgravity, physical parameters such as the interfacial free energy between the crystal and the solution, the distribution coefficient of impurities and the growth rate.

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