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Free-falling Crystals: Biological Macromolecular Crystal Growth Studies in Low Earth Orbit

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Spacecraft orbiting the earth experience a reduced acceleration environment due to being in a state of continuous free-fall. This state colloquially termed microgravity, has produced improved X-ray diffraction quality crystals of biological macromolecules. Improvements in X-ray diffraction resolution (or detail) or signal to noise, provide greater detail in the three-dimensional molecular structure providing information about the molecule, how it works, how to improve its function or how to impede it. Greater molecular detail obtained by crystallization in microgravity, has important implications for structural biology. In this paper we examine the theories behind macromolecule crystal quality improvement in microgravity using results obtained from studies with the model protein, chicken egg white lysozyme.

Introduction

While crystallization can be used as a separation and purification process, the other main driving force behind the crystallization of biological macromolecules is the determination of the three-dimensional molecular structure through the X-ray diffraction analysis of a single crystal. Knowledge of the molecular structure of biological macromolecules provides an understanding of how macromolecules

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function, with direct applications to structure-based drug design and the enhancement of industrial enzymes. In order to obtain the molecular structure one must first grow large (~100 μm), individual, well ordered crystals. The better the crystal quality, then the higher the resolution (or detail) in the diffraction information. More detail increases the accuracy of the molecular structure, reveals information not previously seen, and hence advances the knowledge of the structure.

Spacecraft orbiting the earth are in a condition of continuous free-fall. The reduced acceleration environment experienced in free-fall is commonly referred to as microgravity (meaning 10^{-6} g). The first biological macromolecule crystal growth experiments in microgravity were performed in 1981 using a sounding rocket [1]. In this experiment, observation of the crystal growth process using an on board camera revealed that turbulent convection seen in the ground-based experiments was not observed in the microgravity experiments. This reduction of turbulent flow during crystal growth is postulated to be the underlying process for crystal quality improvement in microgravity. Another aspect of microgravity crystallization, reported recently, is the partitioning of impurities [2, 3]. A review of the literature however, quickly reveals that while some macromolecules show improvement in microgravity others do not [4]. Chicken egg white lysozyme which has been used as a model protein and hence a frequent flyer in the microgravity program, also reflects variation in experimental results with reports of improvement in some studies [3, 5-7], marginal improvement [8] or no improvement [9, 10]. In this paper we address the postulated causes of macromolecule crystal quality improvement in microgravity using observations from lysozyme crystal growth studies.

Buoyancy Driven Convection

In order for crystal growth to occur, macromolecules must be transported to the crystal surface and incorporated into the crystal lattice. Industrial crystallization techniques stir or agitate the bulk solution. Macromolecular crystal growth experiments for structure analysis use unstirred solutions attempting to provide an ordered crystal lattice through slow crystal growth. Under these conditions transport to the crystal surface is initially dominated by molecular diffusion. When

macromolecules are incorporated into the crystal faster than they can be transported to the crystal surface, then a concentration gradient forms around the growing crystal. This concentration gradient is referred to as a depletion zone. The depletion zone also represents a density gradient, as the depleted solution close to the crystal surface is less dense than the bulk solution. The formation of a depletion zone on the ground leads to buoyancy driven convection as the less dense solution close to the crystal surfaces rises in a plume. This phenomenon is illustrated in Figure 1, where a growth plume is observed during the growth of a tetragonal lysozyme crystal [11].

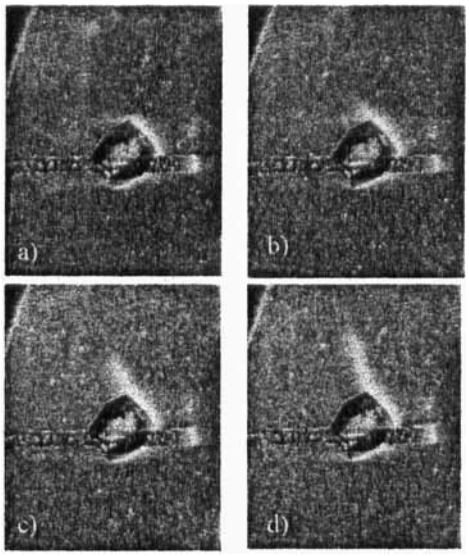

Figure 1. *Schlieren photographs of a growing tetragonal lysozyme crystal (pH 4.0, 0.1M sodium acetate, 5% NaCl, 18°C). The crystal (approximately 1.2 mm in size) was grown on the glass rod used to position it in the observation chamber. The growth plume, shown in white, is seen to form in the successive images (a-d). The time interval between each image is 12 s. The velocity of the growth plume is therefore approximately 30 μm/s.*

In ground experiments the velocity of tetragonal lysozyme crystal growth plumes was found to be between 10 and 50 μm/s, the velocity increasing with increasing supersaturation of the bulk solution. The velocity of the growth plume was also found to increase with crystal size. This convection was postulated to cause the 'cessation of

growth' phenomenon experienced when crystals stop growing even though the solution is still supersaturated. When micro-crystals were exposed to forced solution flow rates (between 18 and 40 μm/s), the crystal growth rate over time was greatly reduced, leading to a termination of crystal growth [11]. This effect of flow is illustrated in Figure 2. In these experiments the solution flow provides a constant bulk supersaturation in the growth cell. The expected growth rate is based on short term crystal growth rates measured at the same bulk supersaturation, in the absence of flow.

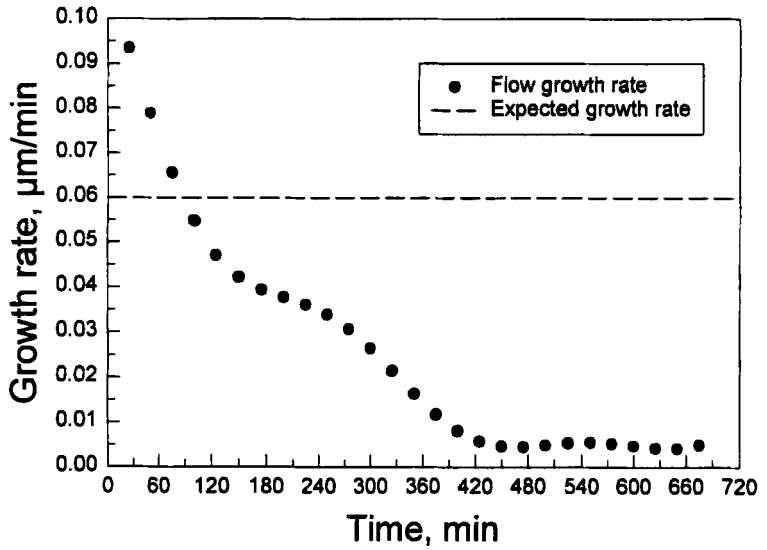

Figure 2. *Effect of solution flow (28 μm/s) on the growth of the lysozyme {110} crystal face. The angle between the direction of flow and the crystal face was 2°. Crystal growth rates were calculated from the data of Pusey et al. [11].*

The negative effects of flow may be due to increased crystal growth rates as more fresh solute is brought to the surface through the action of convection. Over celerity of crystal growth can result in molecules being trapped in high-energy conformations inconsistent with the regular, periodic, crystal lattice. Flow close to the surface may

also poison the crystal surface with mis-orientated or denatured molecules. In microgravity, buoyancy driven convection is not eliminated but it is greatly reduced with the result that depletion zones are more stable. With greatly reduced convective flows more time is available for the growth unit arriving at the growing crystal face to correctly orient and adopt a minimum energy attachment before becoming restricted by subsequent growth. Promotion of a stable depletion zone should therefore result in slower crystal growth providing better ordered, higher quality crystals [2].

In microgravity experiments using highly purified lysozyme solutions, crystals were found to exhibit better X-ray quality characteristics compared to ground controls [5]. This is illustrated in Figure 3. According to current theories [2, 4], in the absence of any detectable impurities, this result is therefore attributed to the reduction in buoyancy driven convection experienced in microgravity.

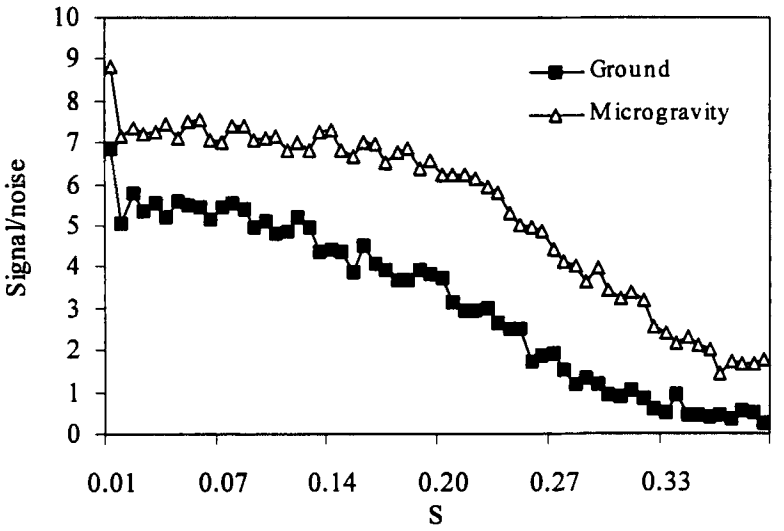

Figure 3. *Signal-to-noise plot for microgravity and ground crystals grown from pure solutions, $S = 4(\sin\theta/\lambda)^2$. Reproduced with permission from Cryst. Growth Des., 2001, 1, 151-158. Copyright 2001 Am. Chem. Soc.*

Lysozyme flow sensitivity however varies with molecular conformation. While tetragonal lysozyme is sensitive to flow, orthorhombic lysozyme does not exhibit a flow sensitivity [12]. The change from tetragonal to orthorhombic lysozyme is

induced by exposure of lysozyme to temperatures above approximately 23°C [13]. This transition has been studied by a number of methods, including C-nuclear magnetic resonance [14], inhibitor binding [15], studies of the activation energy as a function of temperature and pH [16], and X-ray diffraction [17]. These studies all lead to the conclusion that the phase change is due to a subtle structural transition of the protein that results in a change in position of some crystal contacts regions of the molecule [17]. If flow sensitivity can change with different conformations of one macromolecule, then it follows that there is a likely variation of flow sensitivity among the wide variety of biological macromolecules species and conformations within species.

Partitioning of Impurities

Impurities have always been perceived as one of the reasons for lack of success in biological crystal growth experiments [18]. Impurities can be other protein species as well as variations in the macromolecule being crystallized. While impurities may or may not affect the crystal growth rates and morphology [19-22], the key concern is how impurities affect the X-ray crystal quality.

In the case of chicken egg white lysozyme, crystals have been grown in the presence of specific protein impurities, e.g. ovalbumin [22], ovotransferrin [23], turkey egg white lysozyme [23], and the native chicken egg white lysozyme dimer [3, 5]. Only the lysozyme dimer is reported to have a significant negative impact on X-ray crystal quality. The lysozyme dimer appears to be two lysozyme molecules covalently bound, formed during the storage of eggs [24]. Being structurally similar to monomeric lysozyme the dimer is incorporated into the growing crystal in significant quantities [3, 5]. As the dimer is a larger molecule than monomeric lysozyme, it was reported to be favorably partitioned in microgravity crystallization [3], i.e. the dimer incorporation in microgravity crystals was less than that found in crystals grown at the ground. In this case the more stable depletion zones in microgravity are thought to act as mass filters, reducing the incorporation of larger, slowing moving impurities [2, 3]. Accompanying this was the finding that the

microgravity crystals were of a higher X-ray quality. The increase in X-ray quality was attributed to the improved partitioning of impurities [3].

More extensive studies of the partitioning of the lysozyme dimer [5] report no favorable partitioning of the dimer in microgravity The impurity content of both microgravity and ground grown crystals was found to be similar to the impurity concentration in the initial solution. However, while improved partitioning was not observed, an impurity effect was observed in microgravity. For crystals grown in pure solutions, the microgravity crystals were larger and showed improved X-ray quality over ground controls (see Figure 3). As impurity concentration in the initial solution was increased then the diffraction resolution and quality of the microgravity data decreased, becoming similar to that obtained for the ground grown crystals. Figure 4 illustrates the effect of impurity concentration on the signal to noise of the highest resolution X-ray diffraction data. The sensitivity of the X-ray quality of lysozyme to this impurity may explain the variation in crystallization results reported for lysozyme in microgravity experiments, as commercial lysozyme preparations often used in crystal growth experiments contain the lysozyme dimer [25]. From Figure 4 it is interesting to note that the microgravity crystallization is more sensitive to the initial impurity concentration than the earth crystallizations (the pure and highest impurity earth grown samples are not statistically significantly different). Purity it seems is even more important in microgravity crystallization than on the ground. Microgravity was found to offer no significant help in providing useful partitioning of damaging impurities. Improvement in microgravity in this case is a direct result of purification protocols on the ground.

The greater sensitivity of the lysozyme crystallization to impurities in microgravity, observed by Snell et al. [5], may be due to the nature of crystal growth in that environment. With larger and more stable depletion zones the crystal growth rate is dominated by the diffusion of macromolecules to the crystal surface. This provides a slow growth rate and lesser local supersaturation. In ground-based studies with lysozyme we examined the effect of specific impurities (ovalbumin, conalbumin and avidin) on crystal growth rates. Only in the case of conalbumin did we observe an effect and it was only significant at low supersaturation [21]. At high supersaturation

the growth rates in the presence of this impurity were not significantly different to that of the pure material, but as the supersaturation dropped to the mid and low supersaturation ranges there began to be a significant effect. Crystal growth was eventually terminated under conditions where it still flourished with a pure solution. The supersaturation sensitivity of impurities is also confirmed in the impurity crystal growth model of Kubota and Mullin [26], which indicates that impurities become more effective at lower supersaturations. If there is no favorable partitioning, for a system containing damaging impurities and under the same bulk solution conditions, then the microgravity system with its slower crystal growth rates may therefore be more sensitive to the presence of the impurity in comparison to its earth growing counterpart.

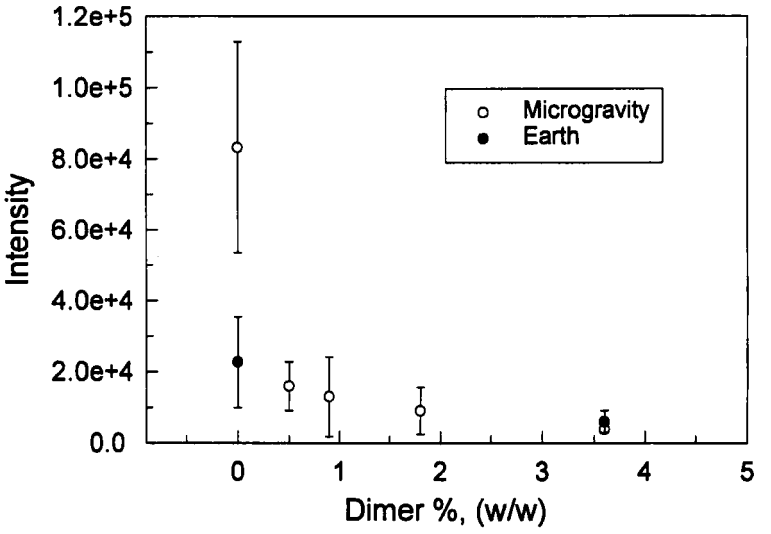

Figure 4. *Intensity plotted against the initial dimer solution concentration for highest resolution data (greater than 2.01 Å). The error bars represent 95% confidence intervals. Reproduced with permission from Cryst. Growth Des., 2001, 1, 151-158. Copyright 2001 Am. Chem. Soc.*

Conclusions

For highly purified solutions the improvement of tetragonal lysozyme crystals in microgravity appears to be due to the reduction in buoyancy driven convection. As increasing incorporation of damaging impurities reduces the X-ray diffraction properties, higher quality crystals can be obtained by preventing the impurity from entering the crystal lattice. The evidence for favorable partitioning in microgravity however is conflicting, indicating that partitioning may be a complex phenomenon. Unless damaging impurities form over time during the crystal growth process, partitioning in microgravity does not have a role that cannot be provided by good biochemical practice on the ground.

Acknowledgements

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Nomenclature

g	Gravitation acceleration	(m/s^2)
S	Scattering vector	(m^{-2})
θ	Bragg angle	(°)
λ	Incident wavelength	(Å)

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