by two-dimensional nucleation at higher supersaturation. A noteworthy outcome of the study was the sensitivity of growth to minor temperature changes. A variation of 2–3 °C was observed to be sufficient to transform the growth mechanism from one regime (spiral growth) to another (by dislocation).

4.1.5.3. *Techniques for evaluating crystal perfection*

The ultimate objective of structural biologists is to analyse crystals of high perfection, in other words, with a minimum of disorder and internal stress. The average disorder of the molecules in the lattice is expressed in the resolution limit of diffraction. Wilson plots provide good illustrations of the diffraction quality for protein crystals. Other sources of disorders, such as dislocations and related defects, as well as the mosaic structure of the crystal, may strongly influence the quality of the diffraction data. They are responsible for increases in the diffuse background scatter and a broadening of diffraction intensities. These defects are difficult to monitor with precision, and dedicated techniques and instruments are required for accurate analysis (reviewed by Chayen *et al.*, 1996).

Mosaicity can be defined experimentally by X-ray rocking-width measurements. An overall diagnostic of crystal quality can be obtained by X-ray diffraction topography. Both techniques have been refined with lysozyme as a test case and are being used for comparative analysis of crystals grown under different conditions, both on earth and in microgravity. For lysozyme and thaumatin, improvement of the mosaicity, as revealed by decreased rocking widths measured with synchrotron radiation, was observed for microgravity-grown crystals (Snell *et al.*, 1995; Ng, Lorber *et al.*, 1997).

Illustration of mosaic-block character in a lysozyme crystal was provided by X-ray topography (Fourme *et al.*, 1995). Comparison of earth and microgravity-grown lysozyme crystals showed a high density of defects in the earth-grown control crystals, while in the microgravity-grown crystals several discrete regions were visible (Stojanoff *et al.*, 1996). X-ray topographs have also been used to compare the orthorhombic and tetragonal forms of lysozyme crystals (Izumi *et al.*, 1996), to monitor temperature-controlled growth of tetragonal lysozyme crystals (Stojanoff *et al.*, 1997), to study the effects of solution variations during growth on the perfection of lysozyme crystals (Dobrianov *et al.*, 1998), and to quantify local misalignments in lysozyme crystal lattices (Otalora *et al.*, 1999).

4.1.6. Use of microgravity

4.1.6.1. *Why microgravity?*

In microgravity, two interrelated parameters, convection and sedimentation, can be controlled. In weightlessness, the elimination of flows that occur in the medium in which the crystal grows theoretically has consequences that may account either for improvements in crystal quality, or crystal deterioration (Chernov, 1997*a*; Carter *et al.*, 1999). The absence of sedimentation permits growth in suspension unperturbed by contact with containing-vessel walls and other crystals. However, protein-crystal movements, some consistent with Marangoni convection (Boggon *et al.*, 1998) and others of diverse origins (García-Ruiz & Otalora, 1997), have been recorded during microgravity growth. On the other hand, it has been proposed that a reduced flow around the crystals minimizes hydrodynamic forces acting on and between the growing crystals and, as a consequence, may favour incorporation of misoriented molecules that act as impurities (Carter *et al.*, 1999). This divergent view of microgravity effects could account for the diversity of results observed in crystallization experiments conducted in this environment, some showing enhanced diffraction qualities of the space-grown crystals (see below), others showing no effect (*e.g.* Vaney *et al.*, 1996) or even decreased crystal quality (*e.g.* Hilgenfeld *et al.*, 1992).

Experiments dealing with the crystallization of proteins and other macromolecules in microgravity have been carried out now for 15 years (DeLucas *et al.*, 1994; Giegé *et al.*, 1995; McPherson, 1996; Boggon *et al.*, 1998). The design of experiments has been based on different strategies. One consists of screening the crystallization of the largest number of proteins, with the intention of obtaining crystals of enhanced quality. In these experiments, monitoring of parameters during growth is restricted, and earth-grown direct control crystals are often not feasible. A second strategic objective is the more thorough study of a few model cases to unravel the basic processes underlying crystal growth. Here, the idea is to monitor as many parameters as possible during flight and, if possible, to conduct ground controls in the same types of crystallization devices, using identical protein samples. In both cases, assessment of the diffraction qualities of the crystals is essential, but precise measurements have only been carried out for the past few years. Altogether, a variety of observations and measurements recorded by many groups of investigators appears to demonstrate, some would say prove, that crystals of biological macromolecules grown in space are superior, in a number of important respects, to equivalent crystals grown in conventional laboratories on earth. This is of some importance, not only from the standpoint of physical phenomena and their understanding, but in a more practical sense as well.

4.1.6.2. *Instrumentation*

Crystallization in microgravity requires specific instrumentation (reviewed by DeLucas *et al.*, 1994; Giegé *et al.*, 1995; McPherson, 1996). A number of reactors have been focused on this goal, some based on current methods used on the ground (batch, dialysis, vapour diffusion), others on more microgravity-relevant approaches, such as free interface diffusion with crystallization vessels of rather large size. The instruments based on this latter method, however, generally cannot be used on earth for control experiments, since with gravity, mixing of the macromolecule and crystallizing-agent solutions occurs by convection. An interesting variation of the classical free interface diffusion system is the hardware using step-gradient diffusion (Sygusch *et al.*, 1996). One of its advantages over more conventional systems is that it provides the possibility of uncoupling nucleation from growth by reducing supersaturation at a constant temperature once nuclei have appeared. A versatile instrument designed by the European Space Agency (ESA) and built by Dornier GmbH is the Advanced Protein Crystallization Facility or APCF (Bosch *et al.*, 1992). The APCF was manifested on a number of US space-shuttle missions and yielded significant comparative 'earth/space' results. It allows monitoring of growth kinetics and can accommodate free interface diffusion (see Fig. 4.1.2.1*d*), dialysis or vapour diffusion. Straightforward ground controls can be conducted with the dialysis cells. A new generation of instruments, the Protein Diagnostic Facility or PCDF, exclusively dedicated to diagnostic measurements of protein-crystal growth, is being developed by ESA and will be installed in the International Space Station (Plester *et al.*, 1999).

4.1.6.3. *Present results: a summary*

Significant and reproducible microgravity experiments have been carried out with a substantial number of model proteins (including lysozyme, thaumatin, canavalin and several plant viruses). The observations in support of microgravity-enhanced crystal growth are primarily of the following nature:

Visual quality and size: The largest dimensions achieved for crystals grown in space were higher than for corresponding crystals

grown at 1*g*. Space-grown crystals were observed to be consistently less marred by cracks, striations, secondary nucleation, visible flaws, inclusions, or aggregate growth. When large numbers of crystals were produced in experiments, morphometric analysis (scoring based on size) of the entire population generally showed a statistically significant tendency toward larger average sizes (*e.g.* DeLucas *et al.*, 1994; Koszelak *et al.*, 1995; Ng, Lorber *et al.*, 1997).

Maximum resolution and Wilson plots: The first quantitative measurements to support conclusions based on visual inspection were those comparing the maximum resolutions of diffraction patterns from corresponding crystals grown on the ground and in space. A striking improvement of resolution was found for paralbumin, where space-grown crystals diffract to 0.9 Å resolution, but earth-grown crystals are not suitable for diffraction analysis (Declercq *et al.*, 1999).

An analytical procedure for comparing X-ray data is the comparative Wilson plot. Reports have appeared in which the maximum obtainable resolution of X-ray diffraction was greater for crystals grown in space than for equivalent crystals produced on earth. Another product of a Wilson plot is the ratio, over the entire resolution range, of the average intensity to the background scatter, taken in small resolution increments across the entire $\sin(2\theta)$ range. This I/σ ratio is, in a way, the peak-to-noise ratio for the measurable X-ray data. Again, as for resolution, the I/σ ratio for X-ray diffraction data collected from crystals grown in space was in several cases reported to be greater than for the corresponding earth-grown crystals [*e.g.* for satellite tobacco mosaic virus (McPherson, 1996) and thaumatin (Ng, Lorber *et al.*, 1997)].

Mosaicity: An additional criterion used to support the enhanced quality of crystals grown in microgravity is the mosaic spread of X-ray diffraction intensities recorded from space- and earth-grown samples. Several reports indicate that for at least some protein crystals (lysozyme, thaumatin), the width and shape of diffraction intensities are improved for crystals grown in microgravity (*e.g.* Snell *et al.*, 1995; Stojanoff *et al.*, 1996; Ng, Lorber *et al.*, 1997).

Impurity incorporation: Impurities can be incorporated in growing crystals and their partitioning between the crystal and the mother liquor shown (Thomas *et al.*, 1998). Based on theoretical considerations, such partitioning should depend on the presence or absence of convection and, therefore, should be gravity-dependent. This is actually the case as demonstrated with lysozyme, for which the microgravity-grown crystals incorporated 4.5 times less impurity (a lysozyme dimer) than the earth controls (Carter *et al.*, 1999).

Crystallographic structure: In a case study with tetragonal hen egg-white lysozyme crystals, a significant improvement of resolution from 1.6 to 1.35 Å resolution, an average decrease of *B* factors, and an improved electron density and water structure have been noticed for the space-grown crystals (Carter *et al.*, 1999).

Altogether, the above examples suggest an overall positive effect of microgravity on protein-crystal growth. To date, however, and because of the youth of microgravity science, in particular in its newest developments, it is not possible to make generalizations for all proteins. Even for the same protein, divergent conclusions can be reached; for example, the quality of the X-ray structure of lysozyme was shown to be improved (Carter *et al.*, 1999) or unaffected by microgravity (Vaney *et al.*, 1996). In this case, the contradiction may originate from different levels of impurities present in the protein batches used in the two studies and/or from non-identical growth conditions in different hardware.

4.1.6.4. *Interpretation of data*

It is conceivable that the alteration of fluid properties by gravity, the occurrence of density fluctuations, or some other property such as these could affect nucleation. Based on computer simulations, it has been suggested that crystals nucleate under different supersaturation and supersaturation rates on ground and in space (Otalora & García-Ruiz, 1997). There is, however, no definitive evidence at this time for how gravity affects nucleation, although it has been observed with thaumatin that the total number of crystals grown in space was less relative to those grown on earth (Ng, Lorber *et al.*, 1997). Gravity expresses itself in fluids, including crystallization mother liquors, by altering mass and heat transport, and it is acknowledged that transport has a real, and in some cases profound, effect on several aspects of growth. It therefore seems reasonable to expect that the growth of crystals is altered once a critical nucleus has formed and that this is important in understanding the effects of microgravity.

Transport would seem to be of particular importance for macromolecular crystallization, because the size of the entities involved requires them to have extremely low diffusivities, two to three orders of magnitude less than for conventional molecules. Elimination of fluid convection may, however, dramatically affect the movement and distribution of macromolecules in the fluid and their transport and absorption to crystal surfaces (Pusey *et al.*, 1988). In addition, most macromolecules, particularly at high concentration, tend to form large non-specific aggregates and clusters in solution. These may very well be a major source of contaminants that become incorporated into the crystal lattices of macromolecules and are, therefore, a major influence on the growth process. By virtue of their size and low diffusivity, the movement of aggregates and large impurities in solution is even more significantly altered. Finally, some macromolecular crystals may grow by the direct addition of three-dimensional nuclei or volume elements of a liquid protein phase, and all macromolecular crystals are, at the very least, affected by these processes. The transport of three-dimensional nuclei or liquid protein droplets, again, by virtue of their size, should be altered in the absence of gravity.

Protein crystals grow in relatively large volumes of mother liquor, hence the consumption of molecules by growing crystals does not significantly exhaust the solution of protein nutrient for a long period of time. Thus, normal crystal growth may proceed to completion at high supersaturation and never approach the metastable phase of supersaturation where growth might proceed more favourably. In earth's gravity, there is continuous density-driven convective mixing in the solution owing to gradients arising from temperature and from incorporation of molecules by the growing crystal. The effects of diffusive transport in the laboratory are almost negligible in comparison to microgravity because of the very slow rate of diffusion of large macromolecules. Because of convective mixing, protein crystals nucleated on earth are continuously exposed to the full concentration of protein nutrient present in the bulk solvent. Convection thus maintains, at the growing crystal interface, excessive and unfavourable supersaturation as growth proceeds. This provides an explanation as to why microgravity may significantly improve the quality of protein crystals. The mechanism for enhanced order and reduction of defects may not be directly due to convective turbulence at growing crystal surfaces, but to reduction of the concentration of nutrient molecules and impurities in the immediate neighbourhood of the growing crystals.

As a macromolecular crystal forms in microgravity, a concentration gradient or 'depletion zone' is established around the nucleus. Because protein diffusion is slow and that of impurities may even be slower, the depletion zone is quasi-stable. The net effect is that the surfaces of the growing crystal interface with a local solution phase at a lower concentration of protein nutrient and impurities than exists in the bulk solvent. The crystal, as it grows, experiences a reduction in its local degree of supersaturation and essentially

creates for itself an environment equivalent to the metastable region where optimal growth might be expected to occur.

4.1.6.5. *The future of crystallization under microgravity*

Several years ago, investigations of macromolecular crystallization under microgravity diverged along two paths. The objective of the first was to produce high-quality crystals for biotechnology and research applications, *e.g.* X-ray diffraction analysis. The crystals themselves were the product, and scientific results were of lesser importance. The goal of the second line of investigation was a definition and description, in a quantitative sense, of the mechanisms by which the quality of crystals was improved (or altered) in microgravity. Understanding and, in the end, controlling the physics of the process was the real objective. This second interest was ably supported by extensive ground-based research based on a variety of sophisticated techniques.

The confluence of results from these two streams has significantly altered prevailing circumstances and attitudes. Persuasive explanations for the observed improvements in size and quality of macromolecular crystals grown in microgravity have emerged, and a convincing theoretical framework now exists for understanding the phenomena involved. Physical methods, such as interferometry and AFM, have revealed the unsuspected variety, structure and density of dislocations and defects inherent in macromolecular crystals. These arrays of defects, which provide the key to the improvement attained in microgravity, have been shown to be far more complex, extensive and dense than those commonly associated with conventional small-molecule crystals. Thus, macromolecular crystals are more sensitive to the unusually high degrees of supersaturation at which they are usually grown and to the mass-transport mechanisms responsible for bringing nutrient to their growing surfaces. The self-regulating nature of protein crystallization in microgravity, through the establishment of local concentration gradients of reduced supersaturation, explains why the diffusive transport that predominates in space produces a significant difference in ultimate crystal quality.

There are currently a number of powerful systems under development by the USA, Europe and Japan. These will be deployed on the International Space Station, where they will form the core facilities for the investigation of macromolecular crystallizations in space. These studies will extend and refine our understanding of the physical principles governing microgravity crystal growth and will better identify the properties of macromolecules likely to benefit most from crystallization in microgravity.

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