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Protein crystallization – is it rocket science?

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Fueled by initial space shuttle results, the National Aeronautics and Space Administration (NASA) has been supporting fundamental studies of macromolecular crystal growth since 1985. The majority of this research is directed at understanding the relationship between experimental variables and important crystal characteristics. The program has resulted in new methods and technology that will benefit the crystallography community's effort to meet the ever-increasing demand for protein structural information. Microgravity crystallization results indicate a potential impact on structural biology's more challenging problems, as soon as long-duration experiments can be performed on the International Space Station.

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▼ NASA (the National Aeronautics and Space Administration, Washington DC, USA) has been supporting a relatively small research program in biotechnology for more than a decade. One component of this research involves the study of the physical and chemical factors influencing macromolecular crystal growth. The crystal growth research was stimulated by results from an experiment designed by a German physicist, Walter Littke, and performed in a microgravity environment on the US Space Shuttle. Two proteins, β-galactosidase and lysozyme, were crystallized using a unique liquid-diffusion crystallization apparatus[1]. Comparison of the space-grown crystals with crystals grown in control experiments on Earth demonstrated a dramatic increase in crystal size and visible quality for the space-grown crystals. These preliminary results suggested that the space shuttle's microgravity (10^{-6} g) environment could be beneficial for macromolecular crystallization.

The effects of microgravity on macromolecular crystal growth

NASA's ground-based, peer-reviewed research program supports studies of the fundamental factors affecting macromolecular crystallization processes. The ground-based research and resultant technology development represent the major emphasis of the NASA program. NASA also supports a small, flight investigator program designed to evaluate the singular effect of microgravity on crystallization. Microgravity results have revealed measurable improvements in X-ray diffraction resolution for ~20% of the proteins crystallized[2–11]. Similar results have been obtained from flight programs sponsored by the European Space Agency (ESA), the Japanese Space Agency (JSA), the Russian Space Agency (RSA), the Chinese Space Agency and the Canadian Space Agency (CSA)[2,12–23]. The theoretical basis behind the hypothesis that a lower gravity level could be beneficial to macromolecular crystal growth is based on three suppositions, which are detailed below.

High-quality crystals grow via diffusive transport

Minimization of potentially harmful buoyant convective flows (experimentally demonstrated to occur in small-molecule and protein crystal growth-solutions in a unit gravity environment) allows crystals to grow via diffusive transport, a mechanism more conducive to the growth of high-quality crystals (Fig. 1)[24,25].

In Fig. 1, Schlieren photography highlights differences in refractive index. The lighter regions indicate lower-density portions of the solution that are therefore flowing upwards with respect to the gravity vector. Convective flow patterns are suppressed without gravitational forces, thereby eliminating the convective plume seen in Fig. 1(a) and (b) and, consequently, creating more uniform conditions across the crystal surface. In microgravity, concentric rings surround the crystal (observed via microscopic interferometry). These

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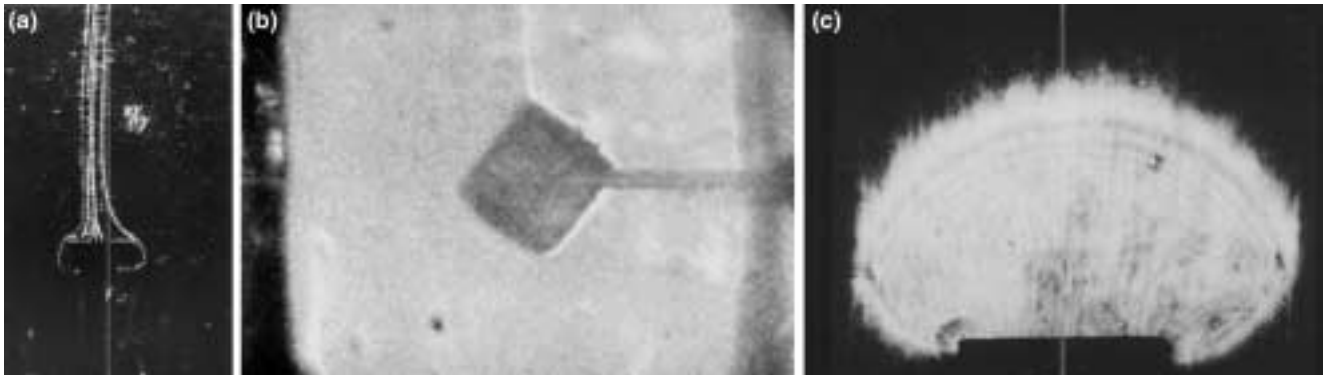

Figure 1. (a) Schlieren photograph of a triglycine sulfate crystal growing under unit gravity at isothermal conditions. **(b)** Schlieren photograph of a hen egg-white lysozyme protein crystal growing under unit gravity at isothermal conditions. **(c)** Interferometric photograph of a triglycine sulfate crystal growing under microgravity at isothermal conditions. Reproduced, with permission, from Refs 24 and 25.

are caused by the depletion of triglycine sulfate and result in slight changes in density as triglycine sulfate is incorporated onto the crystalline surface. More uniform and slower growing conditions allow steady-state diffusion to become the rate-limiting process in the growth of crystals. Microgravity affects transport phenomena that are responsible for the flux of protein molecules from the bulk solution to the growing protein crystal surface. Because of the extremely low diffusion coefficients of protein molecules in aqueous solutions (about 10^{-6}–10^{-7} cm^2 s^{-1}), natural convection caused by concentration gradients within the solution dominates transport on Earth. Convection interferes with the crystal growth process in two principal ways. First, it transports protein molecules to the surface at a rate that is incommensurate with the time-constant required to orient a protein molecule properly for an orderly crystal growth process. Second, because it is so rapid, it does not discriminate between the various protein aggregates (j-mers) that simultaneously exist in dynamic equilibrium in the supersaturated protein solution. The first detriment of convection could thus be simply described as 'jamming' of molecules onto the surface without allowing them sufficient time to reorient themselves into the preferred position for incorporation into the crystal lattice, thereby resulting in a highly ordered crystal. In the nearly zero gravity environment that exists during space flight, diffusion is the only transport mechanism by which molecules in the solution can reach the crystal interface. This slower rate of transport is favorably matched with the time required for reorientation and production of a long-range ordered crystal interface.

In addition, convection interferes with the natural dynamic equilibrium that is established between monomers, dimers, and higher ordered j-mers, in the saturated protein solution. With convective transport, all protein aggregates are presented at the surface, and a complex mix of different basic building blocks at the surface could result in formation of crystal defects that reduce its overall diffraction quality. With diffusion as the only mechanism of transport, a certain matching is established between the rates of transport to the surface, the dynamic equilibrium state of aggregates in the solution and the rate of incorporation into the crystal lattice. For example, monomeric molecules are transported in the solution more rapidly because of their higher diffusivity[5]. A protein monomer in water diffuses at a rate obeying the Stokes–Einstein equation (Eqn. 1):

$$D_{P,W} = \frac{RT}{6\pi\eta_W r_P} \quad (1)$$

In Eqn. 1, $D_{P,W}$ is the hydrodynamic size of the protein molecule in water, R is the universal gas constant, T is the temperature, η_w is the aqueous solution viscosity and r_p is the hydrodynamic size of the protein molecule. A monomer with a diameter of 30 Å has a diffusive coefficient of about 7×10^{-7} cm^2 s^{-1}, about twice as fast as its dimer, and so on. Although it is still unclear whether monomers are always the basic building blocks of the crystal interface, their superior transport rates in near zero gravity, combined with the overall decrease in that rate, provide the setting for orderly growth. The preferred transport of monomers to the crystal surface and the decreasing rate of the entire transport process have been suggested to contribute to the superior quality crystals that are obtained in ~20% of cases in a microgravity environment. Most crystallographers agree that decreasing crystal growth rates often results in higher quality, and sometimes larger, crystals.

Fewer, larger crystals are produced

Minimization of potential nucleation sites as a result of more quiescent growth conditions and quasi-containerless

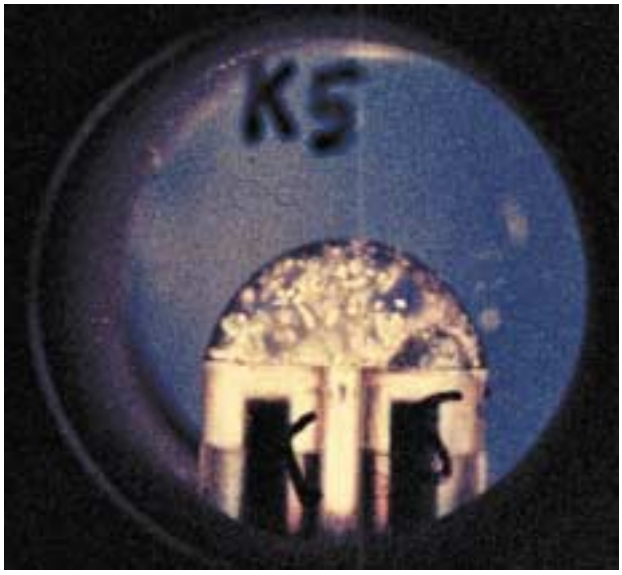

Figure 2. Canavalin crystals grown in microgravity remain suspended within the droplet. Photograph taken on STS-26.

processing can produce fewer but larger crystals. Stirring or vibrating a supersaturated crystallization solution typically induces widespread nucleation, which often results in the growth of several hundred small crystals. In addition, crystals tend to nucleate on surfaces and, as a result, grow attached to a surface, on occasion inducing a lattice mismatch that generally serves to limit the overall diffraction quality of the crystal. In microgravity, solution flows and/or disturbances are dramatically decreased, thereby suppressing unwanted nucleation. Surface tension tends to dominate other forces, allowing experiments to be designed to minimize surface contact (i.e. large protein droplets can be attached to the tip of a syringe).

Uniform growth rates

Microgravity provides a natural environment for uniform growth rates (because of the minimization of convection) and isotropic growth (because of the elimination of crystal sedimentation). On Earth, flows created by buoyancy-induced convection can result in nutrient being delivered unevenly to each of the several different faces on a crystal, thereby causing non-uniform growth[3,5,7]. Protein crystals typically have slightly different densities than that of their growth solution. As a result, the crystals either sediment to the container bottom or float to its surface. In both cases, portions of the crystals will be shielded from the growth medium. This can cause crystals to grow attached to each other or to the surfaces of the container. Microgravity-grown crystals will not float or sediment; instead, they remain suspended within the growth medium, typically yielding single, unattached crystals (Fig. 2).

Crystal defect formation

Several investigators, supported by various space agencies, have used interferometry and atomic force microscopy (AFM) to study nutrient transport and defect formation on macromolecular crystal surfaces[26–34]. Findings suggest that small-molecule impurities and macromolecular aggregates might be responsible for crystal defect formation, which results in decreased crystal quality and size[33–36].

The European Space Agency (ESA) designed an advanced protein crystallization facility (APCF), which provided investigators with the ability to perform *in situ* monitoring of protein crystallization experiments using microscopic video and interferometry. The automated system is capable of monitoring 48 experiments, thereby allowing investigators to observe growing crystals and resultant changes in the protein concentration directly surrounding the crystals. These studies yielded information on the various factors that might affect crystal growth in microgravity and in unit gravity. In addition, the correlation between space shuttle g-jitter (vibrations because of shuttle maneuvers and crew-induced vibrations) and crystal growth-rates[37] could be assessed. There is limited evidence that low-frequency vibrations can be detrimental to macromolecular crystallization[37,38]. On the International Space Station (ISS), investigators can request vibration isolation systems for their experiments. The active rack isolation system (ARIS), developed specifically for the ISS, should dampen vibrations and accelerations induced by the station or its crew.

Fundamental studies of fluid flows induced by localized protein concentration gradients (T. Molenkamp, PhD Thesis, Rijksuniversiteit, Groningen, 1998) that occur in vapor diffusion experiments (in microgravity or in unit gravity) indicate that alternative crystal-growth techniques (i.e. liquid–liquid diffusion or batch crystallization) can provide a more uniform and quiescent environment for solution crystallization experiments. In fact, several investigators have obtained excellent results from microgravity experiments using liquid diffusion as the growth technique (S. Weisgerber, PhD Thesis, University of Manchester, UK, 1993)[5,10,39].

The field of crystallography has experienced several significant advances, such as improvements in X-ray detectors and X-ray sources[40], high-speed computers and enhancements in structure-determination methods[41]. The combination of crystal cryoprotection and new crystallographic techniques allows the determination of a complete protein structure from one crystal in less than a day[41–43]. High-intensity synchrotron sources[43] combined with crystal

cryopreservation methods have reduced the need for multiple crystals of large size. Today, most protein structures can be determined using crystals <200 µm in each dimension. Despite these improvements, the production of crystals of sufficient size and quality continues to be the major impediment to determining protein structures. The overwhelming amount of genomic information that is now available from humans, bacteria, parasites and viruses has resulted in an exponential expansion of proteomic research. Because the biological function of each protein is linked inextricably to its 3D structure, there will be a major increase in the demand for protein structural information. The majority of X-ray structural solutions will continue to result from crystallization in ground-based laboratories and data collection at synchrotrons. However, it is also certain that there will be a significant number of proteins that are either not amenable to crystallization or that produce crystals of insufficient quality for X-ray crystallographic solution. The crystallographic community must continue to explore all approaches in an effort to increase the yield of crystals suitable for X-ray diffraction analysis. Support from space agencies for basic research aimed at improving our understanding of the factors affecting crystal size and quality will continue to enhance experimental success rates on Earth and space.

Although NASA's first microgravity experiment occurred more than a decade ago, the total number of microgravity protein crystallization experiments performed remains extremely small. This is because of the infrequency of US space shuttle flights, the small experiment volume available for macromolecular crystallization and the limited sample capacity in most of the experimental hardware systems. These constraints are compounded by detrimental factors such as the unreliability of space shuttle flights, the short duration of space shuttle flights (most do not exceed ten days), potentially harmful gravitational forces experienced on re-entry, stability of temperature control during sample-transfer operations and other logistical difficulties, such as the amount of time between sample return and the initiation of sample analysis. Despite these difficulties, the space shuttle has proved to be a valuable experimental laboratory, in that it can provide a glimpse of the potential of microgravity to affect biological and physical processes. The ISS will provide a sophisticated laboratory where long duration, iterative biological experiments can be performed. In addition, scientists will be able to analyze crystals via X-ray diffraction, so that crystallization experiments can be optimized or cryopreserved in orbit. High-quality crystals will be cryopreserved robotically for subsequent analysis in ground-based laboratories (i.e. synchrotrons).

The Center for Biophysical Sciences and Engineering (CBSE) at the University of Alabama at Birmingham (UAB) is a NASA Commercial Space Center. As such, the Center has had access to 38 space shuttle flights in support of its microgravity protein crystallization program. This program involves an international co-investigator team located in more than 40 universities and pharmaceutical companies. The key contributions of the co-investigator team are to provide valuable protein samples for microgravity crystallization and to perform the comparative X-ray diffraction analyses of protein crystals using conventional laboratory equipment or synchrotron radiation.

Space experiment protocol

Protein crystallization experiments are extremely variable; identical crystallization conditions using the same batch of purified protein, often yield a wide range of results (i.e. precipitated protein, polycrystalline material, small single crystals or large single crystals). For this reason, the results of CBSE space experiments are always compared with the best X-ray data obtained from a corresponding Earth-grown crystal, regardless of the crystallization method or protein purification batch. However, this method of comparison is not fair, particularly in light of the fact that only one purified protein batch and typically between six and ten experimental conditions are used for the space crystallization experiment. The majority of the proteins chosen for the space experiments have been the subject of years of laboratory work and several different purified protein batches, from which hundreds or thousands of crystallization experiments were prepared, with extensive X-ray data characterization. The best data set from all of the Earth-grown crystals is compared with the best space-grown crystal obtained from one space shuttle flight. The extreme variability of protein crystallization requires these strict comparison guidelines if credible conclusions are to be drawn. If several diffraction-quality crystals for one protein are obtained from a space experiment, co-investigators are encouraged to collect X-ray data from as many crystals as possible so that the crystal-to-crystal variation in diffraction quality can be evaluated. If the space-grown crystals were compared only with control experiments performed on Earth using the identical protein purification batch and experimental conditions, the success rate for the space experiments would be significantly higher.

Results

A comprehensive review of NASA's microgavity protein crystallization results is provided in a report by Kundrot and coworkers[2]. The following are important aspects of the report:

- There was a positive correlation between success rate and the number of times a protein was flown. Eighty-one macromolecules flew only once, experiencing a success rate of <10%. Proteins that received four flight opportunities exhibited enhanced X-ray diffraction resolution in >50% of the cases. For the twelve macromolecules flown on ten space shuttle missions, 100% experienced an improvement in X-ray diffraction resolution or produced crystals that were so large in size that they were amenable to neutron diffraction studies.
- In several examples, the improvements seen in the details of the protein structure were greater than predicted based on the X-ray resolution improvements alone.
- Both poor- and well-diffracting crystals benefited from growth in microgravity.
- In several cases, the total amount of microgravity X-ray diffraction data was more than double that produced by the best diffracting Earth-grown crystals.

Table 1 summarizes results using the vapor-diffusion crystallization apparatus (VDA) for 221 different proteins (these results pertain to the CBSE's program only on 19 missions). On average, each investigator was assigned six experimental crystallization chambers. Investigators were encouraged to screen a range of crystallization conditions centered about the optimum laboratory conditions for the flight hardware. For the vapor-diffusion method, it should be noted that 16.7% of the protein samples did not produce crystals and that 26.2% produced crystals that were too small for X-ray analysis. This occurred despite the fact that any protein chosen for a space flight experiment must be demonstrated on Earth to produce reproducible, diffraction-size crystals in less than seven days using the flight vapor-diffusion apparatus. The explanation for the seemingly poor space results lies in the fact that the crystal nucleation event (initial crystal formation) is substantially delayed in microgravity because of the minimization of convection in the growth solution. In addition, the growth of the crystal subsequent to nucleation is significantly slower because of the decreased nutrient transport to the crystalline surface. If the vapor-diffusion results are evaluated in light of this information, the overall success rate can be viewed as remarkable. The ISS will provide longer growth periods (months) combined with the ability to perform iterative experiments using multiple protein batches. This enhanced capability is expected to produce significantly increased overall success rates and significantly improved magnitude of diffraction resolution.

Table 1. Comparison of space- vs Earth-grown crystals

19 Shuttle missions			221 Macromolecules
No crystals	**Crystals too small for X-ray data collection**	**Diffraction-sized crystals**	**Improved diffraction data**
16.7%	26.2%	57.1%	27.2%

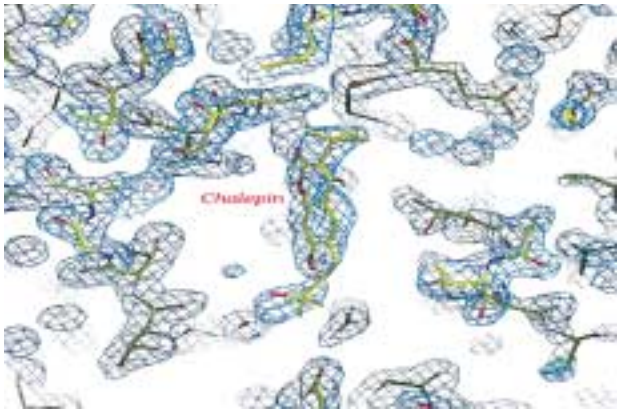

Figure 3. Electron-density map of glyceraldehyde phosphate dehydrogenase–chalepin complex. Clear density for the natural product inhibitor is seen.

One of the more striking microgravity crystallization results involved glyceraldehyde phosphate dehydrogenase (GAPDH) isolated from *Trypanosoma cruzi*, which is a parasite that causes Chagas' disease (G. Oliva, pers. commun.).

Crystals of the protein, complexed with the natural inhibitor chalepin, were obtained in the microgravity environment at 22ºC by vapor diffusion [Space Transportation System (STS)-91]. A diffraction data set was collected at 100 K, to 1.95 Å resolution, and clear electron density for the natural product inhibitor was observed (Fig. 3). This represents the first and only instance where the bound compound was observed in the electron density maps. GAPDH-chalepin complex crystals grown on earth have never revealed the chalepin molecule in electron density maps despite numerous attempts with multiple protein batches. It is not immediately obvious why one experiment in a microgravity environment revealed the structure of the complex crystal, whereas hundreds of attempts on Earth have failed. However, it should be noted that similar results have been observed for other complex crystals grown in microgravity[44,45]. This occurrence could be associated with a combination of the space crystal's diffraction resolution improvements and the improvements in signal:noise ratios for all of the X-ray data, regardless of the resolution range.

Temperature is a thermodynamic variable that sometimes dictates the solubility of a protein in a specified solution[46]. Specific proteins are characterized by increased solubility with increasing temperature, whereas others have decreased, or retrograde, solubility. Because temperature can be controlled precisely, it can be modulated so that protein saturation is approached slowly in a predetermined manner. The formation of fewer nucleation sites and fewer, but larger, superior-quality crystals can result from the slow approach to inducing solution supersaturation. Temperature-induced supersaturation can also be advantageous when combined with the unique microgravity environment of space, where convection currents resulting from temperature change are minimized. Protein samples of bovine insulin, recombinant human insulin and a complex of recombinant human insulin plus a phenol derivative were used as flight samples for temperature-induced crystallization experiments. Crystals were grown by slow cooling from 40 to 22ºC for both forms of insulin. Each ground and flight experiment used identical protein batches, crystallization hardware, activation and growth times and all other experimental conditions. Table 2 summarizes the results from these experiments.

Table 2. Improvements in space-grown insulin crystals

Mission	Protein[a]	Crystal size	Diffraction data characteristics (resolution increase in Å)
STS-37	Bovine insulin	Increased	Increased I/σ(I)
STS-43	Bovine insulin	Increased	0.4 (2.7 → 2.3)
STS-49	Bovine insulin	Increased	0.4 (2.7 → 2.3)
STS-57	Human insulin, R_6	Increased	0.2 (2.0 → 1.8)
STS-60	Insulin, T_3R_3+ phenol	Increased	0.5 (1.9 → 1.4)
STS-86	Insulin, T_6	No change[b]	0.5 (1.5 → 1.0)[c]
STS-95	Insulin, T_6	Increased	0.6 (1.5 → 0.9)[c]
STS-95	Insulin, T_3R_3	Increased	0.4 (1.6 → 1.2)

[a]R_6, T_3R_3, T_6 refer to different forms of human insulin.
[b]Enhanced morphology.
[c]Mosaicity studies showed mg-grown crystals had smaller rocking widths than 1-g-grown crystals.

The objective of the microgravity experiments on STS-57 and STS-60 was to grow crystals of recombinant human insulin and insulin complexes for X-ray analysis and subsequent structure determination; information that will be used for the eventual development of a new long-acting insulin formulation. Although insulin is active in its monomeric form, it is stored as a zinc-complexed hexamer. Phenolic derivatives, by binding the zinc–insulin hexamer, retard its dissolution, thereby slowing bioavailability. A non-toxic phenolic analog–insulin complex could function as improved long-acting insulin. For both missions, samples of the insulin–phenol complex, and subsequently a complex with para (*p*)-hydroxy benzamide (a non-toxic phenol surrogate), produced crystals that were optically and crystallographically superior to their Earth-grown counterparts. The crystals of the insulin–*p*-hydroxy benzamide complex (STS-60) were larger by a factor of two or more (Fig. 4) and were found to diffract X-rays to a significantly higher resolution (Table 2). On STS-60, the experimental hardware included a laser light-scattering system used to monitor the onset of crystal nucleation in real time, so that temperature adjustments could be made in an effort to optimize subsequent crystal growth. The space-grown crystals provided 1.4 Å electron-density maps, which were superior to the 1.9 Å maps from Earth-grown crystals (grown with the identical protein batch and crystallization system). The improved quality of electron-density maps for the space-grown crystals (Fig. 5) enabled researchers to determine the position of the *p*-hydroxy benzamide molecule without ambiguity for the first time. In addition, the electron-density maps generated by the space-grown crystals revealed that there were actually two *p*-hydroxy benzamide molecules present in the binding site rather than one, as originally interpreted from data provided by numerous

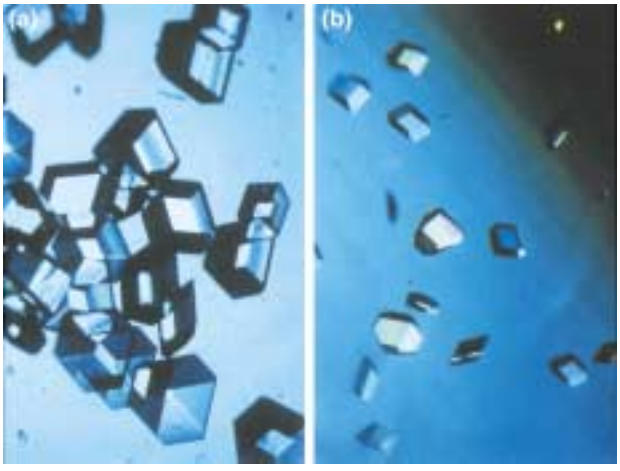

Figure 4. (a) Space-grown crystals of human insulin and **(b)** Earth-grown crystals of human insulin.

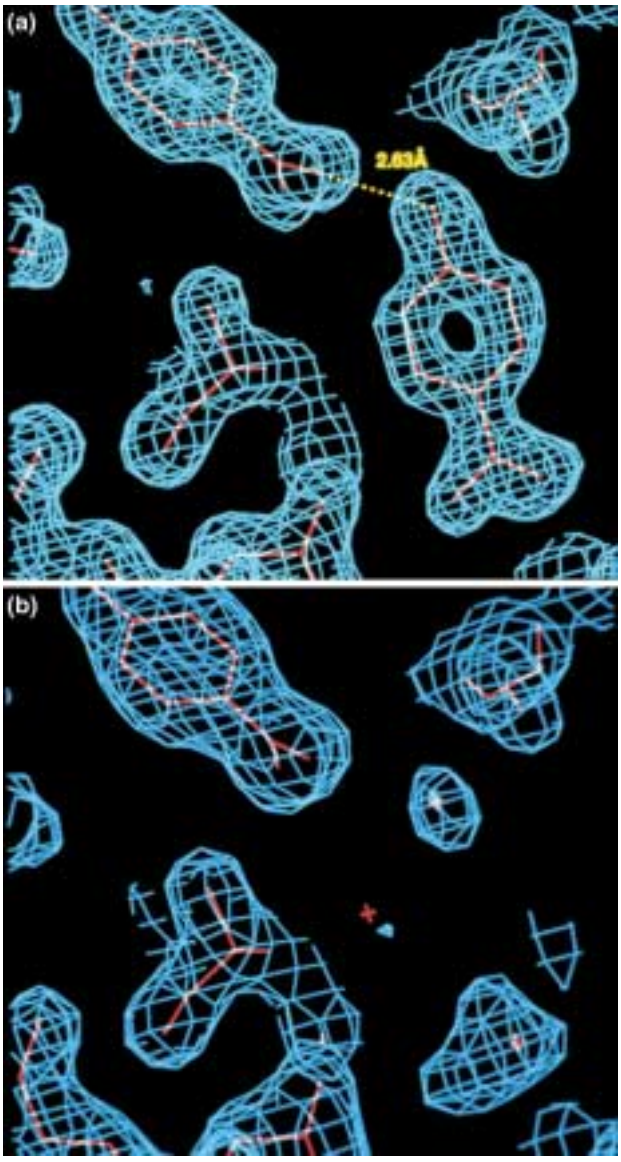

Figure 5. Electron-density maps of **(a)** space-grown insulin and **(b)** Earth-grown insulin.

Earth-grown crystals. The *p*-hydroxy benzamide molecules are involved in an extensive hydrogen-binding pattern between pairs of dimers of the hexamer and two water molecules.

In summary, the X-ray diffraction data from space-grown crystals were superior in every respect to those from Earth-grown crystals and the resulting electron-density maps revealed considerably more detail and completely new information than had been seen in corresponding maps generated from Earth-grown crystals[45]. On STS-95, recombinant human insulin crystals (native protein without phenol compound) were grown, yielding a substantial improvement in the diffraction resolution, 0.9 Å data. A detailed evaluation of the crystal quality not only included the X-ray diffraction resolution but also comparison of individual diffraction peak sharpness (mosaicity), which is related to the molecular order within the crystal (i.e. crystal quality). The microgravity crystals were, on average, 34 times larger, had seven times lower mosaicity, 54 times higher reflection peak heights and diffracted to significantly higher resolution than their Earth-grown counterparts. (E. Snell, pers. commun.). The resolution improvement to 0.9 Å will, for the first time, allow the electronic (as opposed to the geometric) structure of human insulin to be determined. This information will provide valuable insight into the atomic and electronic forces responsible for the insulin–insulin and insulin–water interactions. Mosaicity comparisons reported previously for other proteins exhibited similar improvements for the microgravity-grown crystals[22,23].

The NASA microgravity program was recently reviewed by members of the National Research Council (NRC). Their findings[47] suggest that the microgravity environment could have beneficial effects on protein crystallization. However, the NRC noted that the NASA microgravity protein crystal-growth program produced little impact on the field of structural biology. It is difficult to disagree with this finding. It should be realized, however, that the purpose of NASA's program was to understand the fundamental factors that influence protein crystallization. The program did not support research to determine structures of important biological proteins, unless a particular protein had unique characteristics that might yield new information regarding the fundamental aspects of protein crystallization. In addition, it must be appreciated that researchers typically had access to one shuttle flight and fewer than ten experimental chambers for each protein. NASA has responded to the NRC report by modifying its biotechnology program to stress research directed at important structural biology problems. The ISS will provide a sophisticated laboratory where crystals can be grown for extended time periods and subsequently cryopreserved for analysis at ground-based facilities. It is expected that the enhanced space station capabilities will enable the research community to realize the full potential of microgravity to enhance crystallization and our knowledge of structural biology.

NASA's protein crystal-growth program has resulted in several advances in our understanding of the process of macromolecular crystallization. It has also enabled the identification of factors that affect crystal quality and size, the development of methods to help predict crystallization conditions for new proteins and the development of new crystallization techniques and hardware that improve crystallization in ground-based laboratories.

All protein crystallization methods work by producing an increase in the saturation of the protein such that protein–protein interactions intensify to the point where ordered aggregates of the protein occur. A major impediment in successful crystallization is the ability to find solution conditions that will promote protein interactions that are conducive to crystallization and not to amorphous precipitate formation. Often, thousands of different chemical conditions, and a significant amount of valuable protein, are screened via trial-and-error methods in an attempt to find the correct combination of pH, ionic strength, choice of precipitating (crystallizing) agent, protein concentration, type of buffer and temperature. One exciting discovery by a NASA-supported investigator involves the use of the second virial coefficient, B_{22} (a term associated with static laser light-scattering measurements of dilute protein solutions) as a predictor of probable macromolecular crystallization conditions[48,49]. The combination of a sampling method[50] to increase screening efficiency, and the use of robotic crystallization systems, has increased success rates. However, successful crystallization rates for new purified proteins that are soluble in aqueous solutions remain astonishingly low, typically <30%. Static laser light-scattering of dilute protein solutions provides an unambiguous measure of B_{22}, a thermodynamic term measuring protein–protein interactions[51]. A range of B values that is conducive to crystallization and which corresponds to the presence of a liquid–liquid immiscibility region in the phase diagram has been shown, both theoretically and experimentally, for dilute protein solutions[51,52]. OmpF porin, an integral membrane protein, was recently crystallized using second virial coefficient values to predict solution crystallization conditions[53]. NASA-supported researchers are currently simplifying the experimental procedure to accommodate rapid B_{22} measurements of protein solutions. This will accelerate the determination of initial crystallization conditions while substantially decreasing the requirement for large amounts of purified protein.

Several NASA-funded investigators have theoretically and experimentally demonstrated that control of the pre- and postnucleation phases of macromolecular crystal growth can affect several crystallization characteristics, including crystal size, quality and quantity. Unfortunately, current techniques provide little or no control of the crystallization kinetics. Some of the most common methodologies used for growing protein crystals include dialysis, liquid–liquid diffusion and vapor diffusion. Although the mechanics of these methods are different, the underlying principles of crystallization are identical. In each case, an undersaturated protein solution is prepared. The aqueous solution at this point typically consists of protein, a buffered salt to maintain the desired pH and crystallizing agents. The crystallizing agents, historically referred to as precipitating agents, are commonly inorganic salts, polyethylene glycols or other organic compounds. Initially, the concentration, *c*, of the protein is less than its solubility, *s*, in the specific solution so that the degree of supersaturation or $\sigma = c/s < 1$. The crystallization experiment begins by exposing the initial protein solution to a condition that causes σ to increase. For example, a popular vapor-diffusion configuration, typically described as the hanging-drop method[54], uses a reservoir solution containing buffered precipitant. A buffered protein/precipitant solution 'hangs' from a sealed cover-slip positioned over the reservoir (Fig. 6). The chemical potential of water in the protein solution is higher (has a higher vapor pressure) than that for water in the reservoir and so water is extracted (via vapor diffusion) from the protein solution. As water is removed from the protein solution, the concentration of both the protein and precipitant (crystallizing agent) increases and σ therefore increases. Protein concentration increases rapidly at the onset of the experiment because the chemical potential difference with respect to the crystallizing agent is large. One significant limitation to the traditional vapor-diffusion technique is that the kinetics of the evaporation of water from the protein solution are fixed by the initial concentrations of the solution components. The rate at which the approach to supersaturation of the protein solution occurs cannot be changed by the experimenter, even if modification of the evaporation rate is desirable. In the traditional vapor-diffusion experiment, water diffuses rapidly during the early stages of the experiment and subsequently slows down asymptotically as the experiment progresses. Depending on the solution components, complete equilibration can occur over 3–30 days. Other

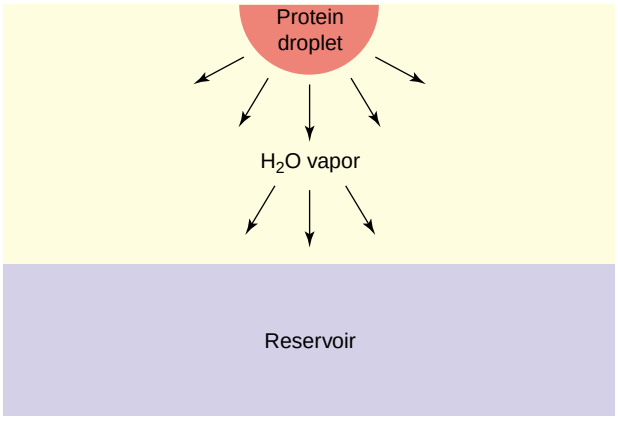

Figure 6. Hanging-drop vapor-diffusion chamber.

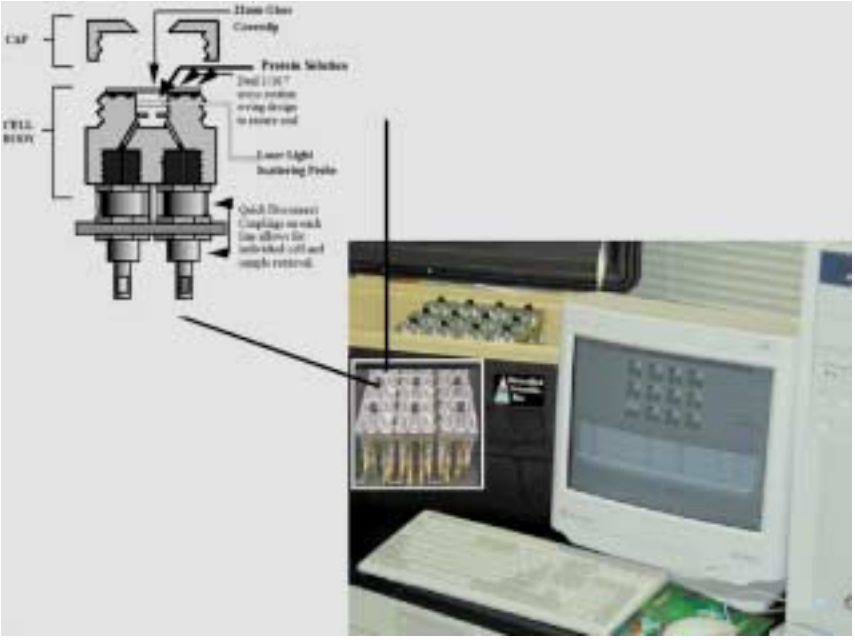

Figure 7. Dynamically controlled vapor-diffusion system.

crystallization methods can give the same result (i.e. increasing σ) but also suffer from the inability to control the process kinetics actively. The CBSE at UAB has developed a dynamically controlled protein crystallization system (DCPCG) that incorporates a controlled flow of dry nitrogen (N_2) gas instead of a reservoir solution to extract water, via computer-control of the vapor phase, from the growth solution[55,56]. A second crystallization system exerts dynamic control over the solution temperature via miniature thermoelectric devices. Using information from noninvasive diagnostics (laser light-scattering and microscopic video), active control of these parameters can, in real-time, affect the supersaturation condition of the protein solution (for both pre-and postnucleation phases). This allows real-time control of the rate of increase for protein supersaturation. The dynamically controlled vapor-diffusion system screens up to 96 different evaporation profiles simultaneously, using chambers in which the protein solution is deployed as a hanging drop (Fig. 7).

Results with these systems indicate that dynamic control of crystallization parameters can have a profound effect on the number and size of crystals obtained for a given protein[55,56]. The dynamically controlled protein crystal-growth system is a new device under development at the CBSE for use on the ISS, as well as in academic and commercial laboratories.

Plans for the ISS include a complete crystallographic laboratory. This facility will support a variety of crystallization hardware systems, an X-ray diffraction rack for crystal characterization or complete X-ray data set collection and a robotically controlled crystal mounting system with automated cryopreservation capabilities[57]. In 1996, the CBSE performed a study for NASA into the feasibility of developing an X-ray facility on the ISS. The results indicated that the concept was feasible, with the exception of three technologies that would require advancement. The first of these was the X-ray source, which would require a reduction in mass, volume and power. Typical systems in ground-based laboratories weigh over 1,800 kg and require up to 10,000 watts of electric power. The second technology improvement involved the development of a robotic system for the selection, harvesting, mounting and cryopreservation of protein crystals. Because of the structural complexity and fragility of protein crystals, all ground-based laboratories rely on the dexterity of the human hand to harvest and prepare crystals for diffraction experiments. The third challenge was to develop an X-ray detector system that did not require hazardous coolants. The CBSE at UAB, in collaboration with companies possessing specific technologies and capabilities, developed and demonstrated an operational laboratory version of the future flight system. The entire facility, designated the X-ray Crystallography Facility (XCF), can be integrated into one international standard payload rack and operated either by the crew, or telerobotically from the ground (Fig. 8). The crystal mounting system is designed to enable both the robotic harvesting of crystals from growth chambers and cryopreservation of these crystals (selected by ground-based scientists via video observation) for automatic on-orbit X-ray analysis. The power and weight requirements of the space station X-ray source were reduced to 30 watts and 23 kg (Ref. 58), while maintaining an X-ray beam of an intensity sufficient for macromolecular X-ray analysis. Another component performs the task of operations management, including monitoring, command and control, and data collection for the integrated rack facility.

Conclusion

One of the unique benefits of space exploration and research is the technology advancements. Technology from the X-ray facility is already finding applications on Earth.

Figure 8. X-ray crystallography facility.

The developer of the X-ray source, Bede Scientific (Durham, UK), is currently marketing a compact, powerful laboratory version for the crystallographic community. A modified form of the robotic crystal harvesting and/or cryopreservation system is being incorporated by Oceaneering Space Systems (Houston, TX, USA) in a crystallography beamline under construction at Argonne National Laboratory (Argonne, IL, USA). It is estimated that automation of the crystal handling will increase sample throughput at least threefold. Protein crystal-growth research has resulted in an improved understanding of fundamental factors that affect macromolecular crystallization, and this information is being used to improve crystallization results. Other significant contributions made by the international space research community include new crystallization methods in gels[58], oils[59–62] and capillaries[63]. However, important questions, such as why crystals stop growing and exactly how crystal quality is affected by buoyancy-induced solution flows, remain unanswered. Fortunately, NASA and other space agencies remain committed to continuing their support of this research. The application of diagnostic tools such as interferometry and atomic force microscopy are providing insight into crystal growth mechanisms and the factors that influence defect formation and growth termination[2,29–36,64,65]. Michelson and Mach–Zehnder interferometry are being used to study the effect of solutal flows around growing crystals on macromolecular transport and attachment kinetics[33,64–66]. Laser light-scattering studies have yielded insight into prenucleation solution conditions, information that can be used to increase the probability of obtaining crystals for new proteins[48,49,51,52]. Clearly, this ground-based research, supported by several space agencies, is providing an improved understanding of the factors influencing macromolecular crystallization. This knowledge will enable investigators to continue to improve crystallization methods for both Earth and space research. No single crystallization method will accommodate all macromolecules. The variety of approaches described here will enable crystallographers to meet the challenge of structural genomics.

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