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# A test of macromolecular crystallization in microgravity: large well ordered insulin crystals

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Crystals of insulin grown in microgravity on Space Shuttle Mission STS-95 were extremely well ordered and unusually large (many >2 mm). The physical characteristics of six microgravity and six earth-grown crystals were examined by X-ray analysis employing superfine $\varphi$ slicing and unfocused synchrotron radiation. This experimental setup allowed hundreds of reflections to be precisely examined from each crystal in a short period of time. The microgravity crystals were on average 34 times larger, had sevenfold lower mosaicity, had 54-fold higher reflection peak heights and diffracted to significantly higher resolution than their earth-grown counterparts. A single mosaic domain model could account for the observed reflection profiles in microgravity crystals, whereas data from earth crystals required a model with multiple mosaic domains. This statistically significant and unbiased characterization indicates that the microgravity environment was useful for the improvement of crystal growth and the resultant diffraction quality in insulin crystals and may be similarly useful for macromolecular crystals in general.

## 1. Introduction

Diffraction data from rhombohedral crystals of hexameric insulin complexed with zinc in a 6:2 ratio were first recorded in 1925 and the first structures of pig insulin were determined 45 y later at 2.8 and 2.5 Å resolution using sealed-tube X-ray sources (Baker *et al.*, 1988, and references therein). To date, the highest resolution structure reported from earth-grown rhombohedral human insulin crystals is at 1.5 Å (PDB entry 4ins; Ciszak & Smith, 1994) obtained with a rotating-anode X-ray source. The $T_6$ form of rhombohedral human insulin crystals was grown in microgravity during the STS-95 Space Shuttle Mission and data beyond 0.9 Å have been collected from cryocooled crystals at a synchrotron (G. D. Smith, personal communication). Microgravity growth is thought to increase the physical perfection and volume of crystals by the reduction of buoyancy, convection and sedimentation effects (Pusey *et al.*, 1986, 1988) and early evidence had shown that growth in microgravity fostered improved order in protein crystals (Snell *et al.*, 1995; Ng *et al.*, 1997). Therefore, the physical characteristics of microgravity and earth-grown insulin crystals were measured in order to explore the reasons why growth in a microgravity environment improved their X-ray diffraction quality.

## 2. Materials and methods

### 2.1. Crystal growth

Crystals were grown in the Commercial Protein Crystallization Facility (PCF; Long *et al.*, 1996) during the nine-day STS-95 Space Shuttle Mission starting October 29, 1998. Crystals were grown by the batch method and nucleation in microgravity was controlled by temperature as described previously by Long *et al.* (1996). This method of growth eliminates the deleterious effects of Marangoni convection seen when vapor-diffusion methods are used in microgravity (Chayen *et al.*, 1997). The earth crystals were grown under identical biochemical conditions at the same time in a duplicate of the PCF apparatus. Prior to data collection, the crystals remained in their unopened PCF bottles at 295 K.

### 2.2. Data collection

In order to minimize instrumental smearing effects, highly parallel and highly monochromatic synchrotron radiation was used at the Stanford Synchrotron Radiation Laboratory (SSRL) bending-magnet beamline 1-5 (Bellamy *et al.*, 2000). To provide a statistically valid number of measurements in a reasonable amount of beamtime, data were collected with

**Table 1**
Diffraction statistics.

| Sample | Date† | Orthogonal crystal dimensions (mm) | Crystal volume ($mm^3$) | Average maximum intensity‡ (counts) | Average $\eta$§ (°) | No. of reflections | No. of data frames |
|---|---|---|---|---|---|---|---|
| Earth-grown insulin crystals | | | | | | | |
| Earth-1 | 12/98 | 0.35 × 0.35 × 0.32 | 0.04 | 344 | 0.031 (0.017) | 170 | 2000 |
| Earth-2 | 12/98 | 0.34 × 0.26 × 0.13 | 0.01 | 880 | 0.035 (0.015) | 20 | 500 |
| Earth-3 | 12/98 | 0.40 × 0.27 × 0.19 | 0.02 | 914 | 0.017 (0.005) | 174 | 2000 |
| Earth-4 | 7/99 | 0.43 × 0.34 × 0.19 | 0.03 | 81 | 0.038 (0.024) | 14 | 2000 |
| Earth-5 | 7/99 | 0.39 × 0.24 × 0.22 | 0.02 | 236 | 0.013 (0.004) | 172 | 1999 |
| Earth-6 | 7/99 | 0.39 × 0.24 × 0.17 | 0.02 | 172 | 0.023 (0.010) | 72 | 2000 |
| Microgravity-grown insulin crystals | | | | | | | |
| $\mu$g-1 | 12/98 | 0.96 × 0.88 × 0.37 | 0.31 | 7510 | 0.004 (0.002) | 502 | 2000 |
| $\mu$g-2 | 12/98 | 1.20 × 0.72 × 0.48 | 0.42 | 7811 | 0.006 (0.005) | 241 | 1000 |
| $\mu$g-3 | 12/98 | 0.90 × 0.88 × 0.32 | 0.25 | 8195 | 0.004 (0.004) | 176 | 500 |
| $\mu$g-4 | 7/99 | 1.29 × 0.84 × 0.43 | 0.47 | 12846 | 0.002 (0.001) | 491 | 2000 |
| $\mu$g-5 | 7/99 | 1.72 × 1.31 × 0.90 | 2.04 | 8362 | 0.004 (0.002) | 489 | 2000 |
| $\mu$g-6 | 7/99 | 1.59 × 1.59 × 0.50 | 1.25 | 7155 | 0.003 (0.001) | 447 | 2000 |

† For the 12/98 data, $\Delta\lambda/\lambda = 2.43 \times 10^{-4}$, vertical beam divergence ($\sigma_v$) = 19.5 µrad and horizontal beam divergence ($\sigma_h$) = 48.0 µrad. For the 7/99 data, $\Delta\lambda/\lambda = 1.94 \times 10^{-4}$, $\sigma_v$ = 15.5 µrad and $\sigma_h$ = 43.6 µrad. ‡ The average peak height normalized for a 2 s exposure time is reported. § Standard deviations are given in parentheses.

a Quantum-4 CCD detector (ADSC) using the rotation camera geometry.

Overall, the microgravity-grown crystals were larger and contained fewer visible flaws than their earth-grown counterparts (Fig. 1). The majority of the earth-grown crystals had sedimented to the bottom of the growth chamber and grew as clusters of many crystals. Most of the large earth crystals were clustered. The best-looking single crystals were chosen for mounting. The population of microgravity crystals consisted of many large single crystals. Presumably, the lack of crystal clusters in the microgravity samples was because the crystals did not sediment during growth in microgravity. The microgravity crystals for study were chosen at random; if anything, the study was biased in favor of the earth crystals. Visually flawless crystals, six from microgravity and six from earth, were mounted in 1 mm quartz glass capillaries (Table 1). The larger microgravity crystals were mounted near the bell of the capillary. Three crystals of each type were mounted and exposed in the first data-collection period, approximately one month after the return of the mission; the remaining six crystals were mounted and exposed approximately six months later. Crystals were stored in their original growth hardware at 295 K until they were used. During data collection, the crystals were maintained at 295 K with a regulated gas stream.

The following data-collection strategy was used. To determine the crystal orientation, two orthogonal 8–10° swaths were collected ($\Delta\varphi = 1°$ with 60 s exposure) and processed with *MOSFLM* (Powell, 1999). A 1.0° image was then selected from each swath and superfine $\varphi$-sliced data (stills spaced by 0.001° with 2 or 5 s exposure time depending on the diffraction strength of the crystal) corresponding to that image were collected for mosaicity measurements. The crystal-to-detector distance was 170 mm and the beam was collimated to 0.3 mm in diameter. The space group was *R*3, with unit-cell parameters $a = b = 82.8$, $c = 34.2$ Å.

The reflections were profiled in the $\varphi$ (rotation) dimension as described in Bellamy *et al.* (2000). It was not feasible to profile them in additional dimensions from the spot shape on the detector because the detector resolution was inadequate. Under the conditions used, each detector pixel subtended more than 0.054°, more than 50 times the resolution obtained in the $\varphi$ dimension.

### 2.3. Data processing

Data were processed using *BEAM-ish* (Lovelace *et al.*, 2000) and the true crystal mosaicity ($\eta$) was deconvoluted from the measured reflection full-width at half-maximum ($\varphi_R$) as described previously (Bellamy *et al.*, 2000). Data from the earth-grown crystals were much weaker overall than the data from the microgravity-grown crystals. To reduce noise, the earth data were smoothed by averaging the data in a 0.003° window. This was not necessary for the microgravity data. In order to be accepted for profile analysis, the reflections from the earth crystals had to have $I_{max} > 100$ and $I_{max}/I_{ave} > 5$. For the microgravity crystal the thresholds were more stringent, with $I_{max} > 150$ and $I_{max}/I_{ave} > 10$. In both cases, $I_{ave}$ was defined as the average of all the integrated spot intensities at the reflection's location measured during the fine-$\varphi$ collection after removal of the 'zingers'. Zingers are signals produced in the detector by cosmic rays or radioactive decay. This is clearly not the standard method of obtaining accurate reflection intensities, but it is computationally simple and suffices to identify the reflections strong enough to provide statistically reliable data.

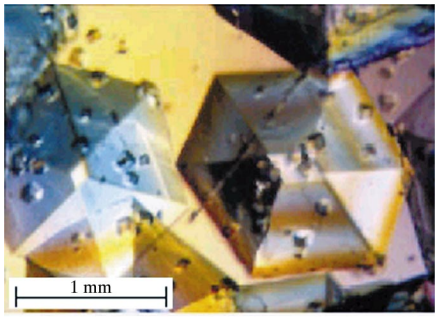
## 3. Results and discussion

When compared with their earth-grown counterparts, microgravity-grown crystals are extremely well ordered. Reflection profiles from microgravity crystals were best fit by a single Gaussian function, whereas earth crystals required several Gaussians (Fig. 2). Therefore, the microgravity crystals appear to be best described as composed primarily of one resolvable mosaic domain and the earth crystals of several domains. This improvement in internal order was found in all microgravity samples studied.

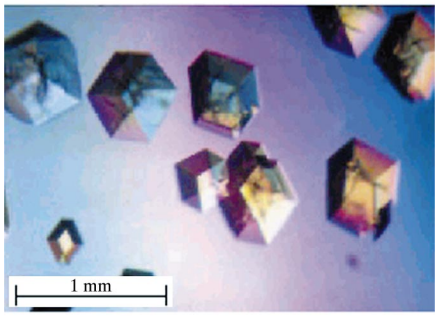
The microgravity crystals diffracted strongly and between 447 and 502 reflections were profiled in 2° of superfine $\varphi$-sliced data. This is to be compared with 14–174 reflections profiled in equivalently accumulated data from the earth crystals (Table 1). The disparity in the numbers of reflections profiled is a consequence of the relative paucity of reflections strong enough to be accurately profiled in the earth data. Overall, the microgravity crystals were of very similar quality, whereas the quality of the earth crystals varied significantly (Table 1). The best microgravity crystals ($\mu$g-4 and $\mu$g-6) had an average $\eta$ of 0.002 and 0.003°, respectively, each with a standard deviation of only 0.001°. It is noteworthy that these values are near the limit of resolution of the instrument configuration used (Bellamy *et al.*, 2000). Two of the earth crystals (earth-5 and earth-3) had fairly good mosaicity with average $\eta$ values of 0.013° (s.d. = 0.004°) and 0.017° (s.d. = 0.005°), respectively, yet these $\eta$ values were 6.5 and 8.5 times higher than the best microgravity crystals, and both crystals were relatively poor diffractors (see Table 1 and Fig. 3*b*). For any given earth crystal, the $\eta$ values for individual reflections varied over a surprisingly large range, with standard deviations of 0.004–0.024°. The spread in $\eta$ for microgravity crystals was four to five times narrower, with standard deviations ranging from 0.001 to 0.005°. It is noteworthy that three of the earth-5 $\eta$ values overlap with the $\mu$g-4 reflections (Fig. 3). This illustrates the importance of collecting a statistically

significant number of reflections from each sample in that if only these three reflections had been measured the samples would have been falsely concluded to be indistinguishable. A Student's $t$ test on the microgravity and ground mosaicity values showed normal distributions but unequal variance in the two populations. This is reflected in the greater standard deviations for the earth data. A non-parametric distribution-free Mann–Whitney rank sum test confirms that the microgravity and the earth data are statistically different from each other at the 99% confidence interval ($T = 57$, $p = 0.002$). It is important not only to collect a statistically significant number of reflections, but also to collect from a statistically significant number of samples.

The improvement in internal order in combination with the increase in the volume of crystal illuminated by the synchrotron beam resulted in dramatically more intense diffraction from the microgravity crystals. For example, the microgravity crystal with the best mosaicity, $\mu$g-4, was 23 times larger than the best earth crystal, earth-5, had 6.5-fold lower mosaicity and had 54-fold higher reflection peak heights (Fig. 3*b*, Table 1). The largest earth crystal, earth-1, had 24-fold lower reflection peak heights and 7.8-fold higher mosaicity and was 6.3 times smaller than the smallest microgravity crystal studied, $\mu$g-3. The worst microgravity crystal, $\mu$g-2, had 2.2 times lower mosaicity than the best earth crystal, earth-5, but was 21 times larger and had reflection peak heights that were 33 times higher. The earth crystals were fully immersed in the beam, whereas the larger microgravity crystals were only partially illuminated. This makes it difficult to quantitatively compare diffraction strength; however, while microgravity crystals were significantly larger than the earth-grown counterparts, the increase in the maximum reflection peak height from the microgravity crystals is greater than the increase in the illuminated volume of the crystal. There is no evidence for any handling problems during the mounting as evidenced by the normal distribution of the results and two clear sample populations. The lack of sample degradation between the two synchrotron runs is shown in Table 1. Storage in the original growth container at the growth temperature seems adequate for insulin.




(*a*)




(*b*)

**Figure 1**
Representative insulin crystals: (*a*) microgravity grown and (*b*) earth grown.

## 4. Conclusions

The first studies showing that microgravity reduces mosaicity (Snell *et al.*, 1995; Ng *et al.*, 1997) were confirmed. The diffraction signals from the microgravity crystals were much cleaner than from the earth crystals (Fig. 2 and Fig. 3) owing to the reduction in mosaicity. Microgravity can provide an environment where large internally well ordered crystals can be grown. Such crystals provide better spatial resolution, less spot overlap and a higher signal-to-noise ratio. Large unit cells with spatial overlap problems, Laue studies and emerging *ab initio* phasing technologies that require accurate intensity data, such as sulfur anomalous scattering (Wang, 1985), or require low mosaicity, such as triplet-phase measurements using

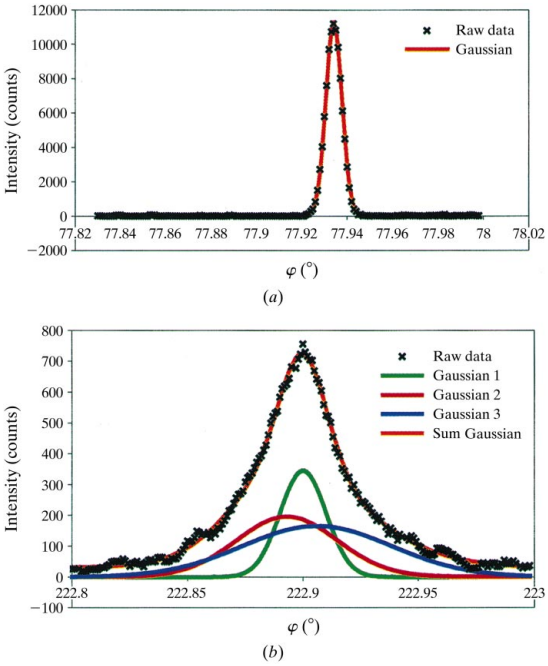



**Figure 2**
Typical reflection profiles for (*a*) the (−14, −9, −2) reflection from crystal $\mu$g-4 ($\varphi_R = 0.010°$, $\eta = 0.004°$) and (*b*) (5, −16, 3) reflection from crystal earth-5 ($\varphi_R = 0.036°$, $\eta = 0.010°$; see Table 1).

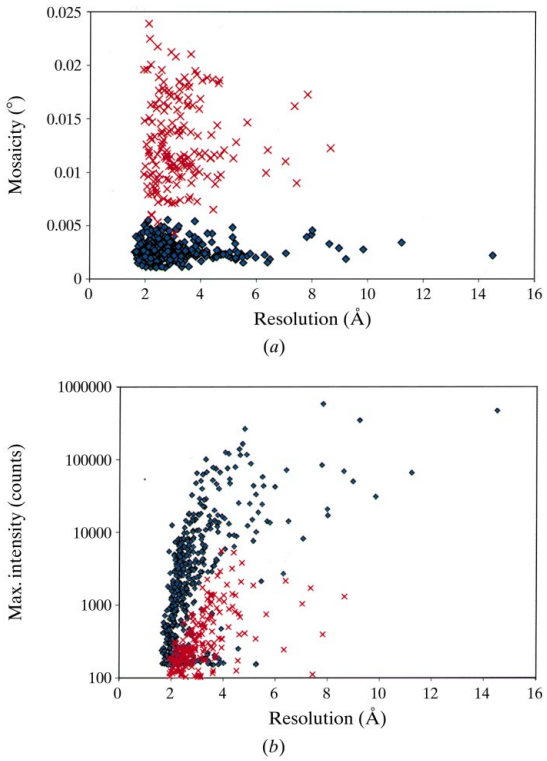



**Figure 3**
Crystal-quality comparison of the most perfect microgravity ($\mu$g-4, blue diamonds) and earth crystals (earth-5, red crosses) by plotting individual reflection (*a*) mosaicity and (*b*) background-subtracted maximum intensity against resolution. The data were cut off at the detector edge. For the microgravity crystals, the data extended beyond this limit. Maximum intensity normalized to 2 s X-ray exposure is plotted on a log scale.

reference-beam X-ray diffraction (Shen *et al.*, 2000; Weckert & Hummer, 1997), will benefit significantly from such high-quality crystals.

Large crystals of macromolecules are often imperfect, which results in poor diffraction quality. Partly because of this, the current trend is to collect data from small crystals using synchrotron radiation. Microgravity appears to be able to break the common inverse relationship between crystal size and crystal quality. It may also enable diffraction-quality crystals to be grown where previously only microcrystals could be obtained. Excellent crystals of large volume will enable more samples to be studied using neutron diffraction. Neutron studies require large-volume crystals, $>1$ mm$^3$, owing to the weak scattering and low beam intensity. In addition, when the Laue method is used, reflection overlap is a problem that can be greatly reduced with low mosaicity. This last requirement is essential for samples with large unit cells.

In this test case, microgravity had a dramatic effect on the size and physical perfection of insulin crystals grown by a temperature-regulated batch method. It is noteworthy that the growth method employed essentially eliminates the Maragoni convection that, in principle, affects other growth methodologies. Six crystals from microgravity and six from earth were studied. The results from microgravity and earth were self-consistent and distinct. Each result is based on many individual reflections. In this case, microgravity passed the test.

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