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Improved diffraction of antithrombin crystals grown in microgravity

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Abstract

Crystals of antithrombin were grown both on earth and in microgravity aboard US Space Shuttle Flight STS-67. The quality of crystals grown in both environments was highly variable and many could not be indexed. The microgravity crystals, however, generally diffracted better, as demonstrated by a novel procedure that estimates the resolution of the Bragg scatter from single diffraction images, without requiring knowledge of the cell dimensions of the crystal. Whereas the best earth-grown crystals never diffracted beyond 3 Å resolution, the best microgravity crystal diffracted to 2.6 Å. The improvement, demonstrated here by a comparison of 23 microgravity and 12 earth-grown crystals, is attributed to better ordered crystal growth in microgravity, although other factors may have contributed also.

1. Introduction

Antithrombin crystals previously grown on earth had a diffraction limit of 3.0 Å (Wardell, Abrahams, Bruce, Skinner & Leslie, 1993; Carrell, Stein, Fermi & Wardell, 1994) because of disorder in the crystal lattice. We, therefore, performed a series of protein crystal growth experiments on US Space Shuttle flight STS-67, in March 1995, in an attempt to derive better ordered crystals. Fully active antithrombin was either purified from freshly frozen human plasma (McKay, 1981) or was regenerated from commercially purchased antithrombin concentrate (Bio Products Laboratory, Hertsfordshire, UK) by passage over heparin–Sepharose and then Q-Sepharose anion-exchange chromatography. Regenerated material had functional and crystallization properties indistinguishable from antithrombin isolated directly from freshly frozen plasma. Protein was prepared within one week of the scheduled launch date of the mission and maintained at 277 K until being loaded into a new Protein Crystallization Apparatus for Microgravity (PCAM; Carter, 1995). The PCAM hardware (to be described in detail elsewhere) uses a sitting-drop vapor-diffusion technique. The reservoir solution is contained in an absorbant wick to prevent mixing with the protein solution in microgravity and to prevent splashing during takeoff and landing. An elastomer seal, used to separate the sitting drops from the reservoir solution, is retracted to allow their equilibration once the PCAM's are in a microgravity environment. Crystals appeared in similar conditions to those reported earlier (10–20 mg ml^{-1} protein, 18–19% PEG 4000, 50 m*M* Na/KPO_4, 0.05% NaN_3, pH 6.7; Wardell *et al.*, 1993). The crystals contain two molecules per asymmetric unit; one in an active conformation and one inactive as previously reported (Wardell *et al.*, 1993; Schreuder *et al.*, 1994).

Control crystals were grown on earth for comparison with those produced in microgravity, with some differences. Because of a mechanical failure during protein isolation, the yield of antithrombin was only sufficient for the microgravity crystallization trials, and earth-grown control crystals were produced with protein isolated at a different time from plasma or from the same batch of commercial antithrombin concentrate. The chemicals used in the crystallization buffers were identical, but the deionized water used to make up the solutions was from a different source. The earth-grown controls were produced using the same vapor-diffusion technique, but using plastic rather than the special elastomer to seal the trays. Microgravity crystallization took place in a dark sealed container thermostated at 294 K, whereas the earth-grown controls were contained in a room occasionally exposed to fluorescent light and where the temperature of 294 K was less stringently regulated. Not all of the control experiments made use of the absorbent wicks used exclusively in the microgravity trials to contain the reservoir solutions. Earth crystals appeared in 10 d and continued to appear and grow for a further 10 d (Wardell *et al.*, 1993). Assuming a similar growth time, it would be expected that many of the microgravity crystals would not have completed growth within the 16 d duration of the mission, and they were visibly slightly smaller than the earth-grown controls. Because of the limited beam time available at the synchrotron, no systematic measurements of crystal volume were taken before the diffraction experiments. Representative crystal volumes derived from crystals used to make a photographic record of the mission were 1.52×10^{-3} mm^3 for earth crystals and 1.22×10^{-3} mm^3 for microgravity crystals. Both batches of crystals were handled identically and we selected the best crystals for diffraction analysis at 100 K.

Before freezing in liquid nitrogen or in a 100 K nitrogen stream, crystals were soaked for 5–30 s in a cryo-protectant buffer [25% 2-methyl-2,4-pentanediol (MPD), 20% PEG 4000, 50 m*M* Na/KPO_4, pH 6.7]. In search of an untwinned crystal with a low mosaicity, we diffracted 23 flash-frozen microgravity crystals and 12 flash-frozen earth-grown crystals at station 7.2 of the Synchrotron Radiation Source in Daresbury, UK, using a wavelength of 1.488 Å. Visual inspection of the diffraction images gave the impression that the microgravity crystals were generally of a higher quality than those grown on earth.

In order to test this hypothesis, one of us (JPA) developed an algorithm for estimating the resolution of the Bragg scatter of twinned or highly mosaic crystals that cannot be indexed. Radial averaging, after exclusion of outliers, provides an inaccurate estimate of the local background in the presence of anisotropic diffuse scatter. Instead, the local background was calculated by Fourier filtering. After a first Fourier transform, the values are set to zero of reciprocal pixels that are more distant from the origin of the transform than 0.02 times the size

of the reciprocal image. Reciprocal pixels closer to the origin are scaled as follows,

$$P_{new} = P_{old}[1 - (d_p/0.02)], \quad (1)$$

where P_{new} is the new value of the reciprocal pixel, P_{old} is the old value of the reciprocal pixel and d_p is the distance of the reciprocal pixel from the origin of the transform, relative to the size of the transformed image.

After back transformation, the filtered image is assumed to give a first approximation of the local background. In the original image, pixels are replaced by the estimated local background if their value is larger than six times the standard deviation (based on counting statistics) of the background. The resulting image is filtered less stringently by replacing the relative distance of 0.02 in (1) by 0.2. After back transformation, details of the size of diffraction spots have disappeared and the filtered image is assumed to represent the smoothly varying diffuse background of the original diffraction image. The first coarse Fourier filter removes intense spots that would otherwise cause ripples due to series termination after the second finer filter. The relative distances of 0.02 and 0.2 times the size of the reciprocal image in (1) determine the amount of detail removed. These values give good results for patterns with sharp diffraction spots measured with the same detector. Visual inspection shows that all Bragg scatter is removed without obviously affecting the diffuse background.

A plot of the skewness of the original intensity distribution relative to the calculated background, as a function of resolution then gives a quantitative estimate of the quality of the diffraction image. Skewness is a statistical measure of the asymmetry of a distribution and is calculated by

$$\text{Skew} = [1/N \sum_j (P_j - b)/\sigma]^3, \quad (2)$$

where N is the number of pixels in a resolution bin, P_j is the value at pixel j of the original image in the resolution bin, b is the background of pixel j as determined from the filtered image, and σ is the root-mean-square of the difference between the pixel values and their calculated background values.

If the signal of the background is sufficiently high to be approximated by a symmetrical Gaussian distribution, the skewness should be higher than 1 in those resolution bins in which Bragg scatter occurs. Fig. 1 demonstrates the application of the procedure to diffraction images of both the best

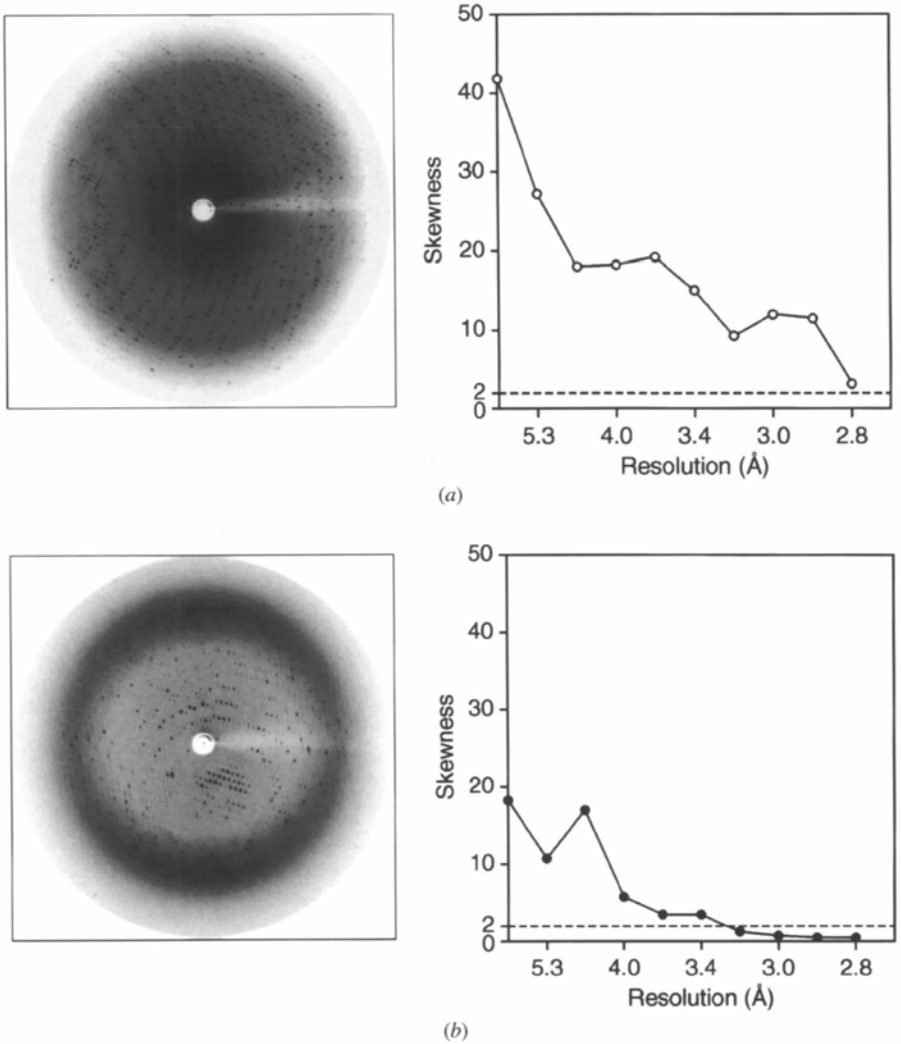

Fig. 1. 1° oscillation diffraction images (outer resolution limit of 2.6 Å) and corresponding skewness *versus* resolution plots for (*a*) the best microgravity-grown crystal and (*b*) the best earth-grown crystal.

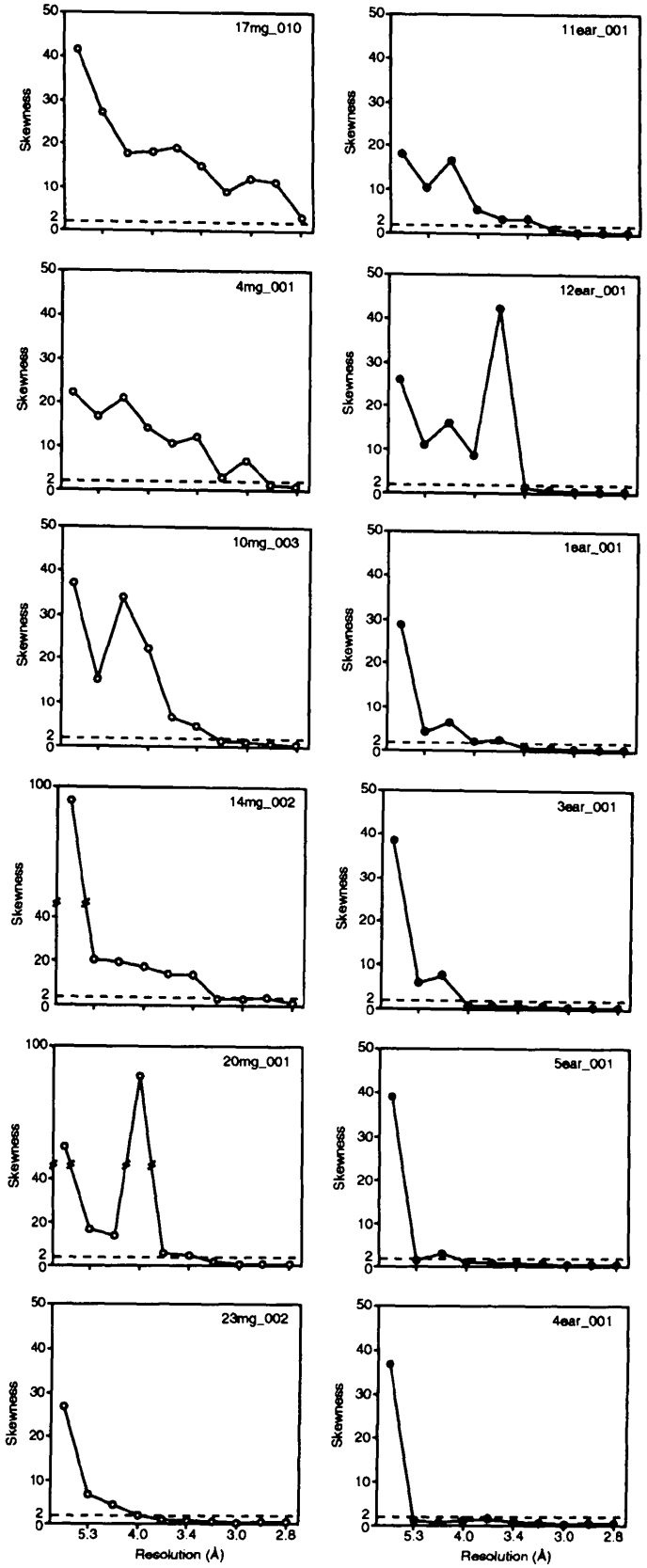

Fig. 2. Plots of skewness *versus* resolution for images from six microgravity-grown (left) and six earth-grown (right) crystals

microgravity and earth crystals grown in the PCAM device. The quality of diffraction of the microgravity crystal is substantially higher than that of the earth-grown crystal and this is borne out quantitatively in the corresponding skewness *versus* resolution plots. These show that the microgravity crystal diffracts beyond 2.8 Å, whereas the earth-grown crystal only diffracts to 3.2 Å. We attribute the absence of obvious Bragg scatter in resolution bins with a skewness higher than 1, but lower than 2 to the Poissonian, rather than Gaussian nature of counting statistics.

In order to give a representative sample of their diffraction characteristics, six earth-grown and six microgravity crystals were randomly selected, and their skewness *versus* resolution plots are shown in Fig. 2. Again, these graphs clearly indicate that the microgravity crystals, on average, diffract better, and to a higher resolution than the earth-grown crystals.

2. Discussion

Until now we have been unsuccessful in extending the resolution of antithrombin crystals beyond 3 Å despite exploring procedures including; additional protein purification using preparative isoelectric focusing; deglycosylation; using an equal mixture of active and latent antithrombin in the crystallization drops; growing different crystal forms; using various additives; and trying different crystallization techniques. The best earth-grown crystals diffracted to 3 Å and were grown by microdialysis.

In several well documented cases, microgravity has been reported to significantly enhance protein crystal growth (DeLucas *et al.*, 1989; Day & McPherson, 1992). Microgravity abolishes the convective flow that produces turbulence at the faces of growing crystals (Velicov, Alexander & Rosenberg, 1996) which can lead to lattice dislocations and other imperfections. In addition, sedimentation of growing crystals, which can interfere with the formation of single crystals, is eliminated in the absence of gravity.

Here we demonstrate that small crystals of antithrombin grown in microgravity by vapor diffusion diffract better than any of the large number of earth-grown crystals we have ever examined. In the absence of unequivocal controls we cannot totally exclude the contribution of other unknown factors to the improvement. However, in light of our numerous unsuccessful previous efforts to improve antithrombin crystal quality, and the successful outcome of the first microgravity experiment, we feel confident that the absence of gravity contributed significantly to the improvement seen in diffraction. Recently, data from a microgravity crystal has allowed us to extend the resolution of the antithrombin structure to 2.6 Å, which has given the first insights of the structural basis for the switching between high and low heparin-affinity states in antithrombin (Skinner *et al.*, 1997).

References

Carrell, R. W., Stein, P. E., Fermi, G. & Wardell, M. R. (1994). *Structure*, **2**, 257–270.

Carter, D. C. (1995). *NASA Technical Memorandum 4677. Microgravity Science & Applications*, pp. 26–27. Washington DC: NASA.

Day, J. & McPherson, A. (1992). *Protein Sci.* **1**, 1254–1268.

DeLucas, L. J., Smith, C. D., Smith, H. W., Vijay-Kumar, S., Senadhi, S. E., Ealick, S. E., Carter, D. C., Snyder, R. S., Weber, P. C., Salemme, F. R., Ohlendorh, D. H., Einspahr, H. M., Clancy, L. L., Navia, M. A., McKeever, B. M., Nagabhushan, T. L., Nelson, G., McPherson, A., Koszelak, S., Taylor, G., Stammers, D., Powell, K., Darby, G. & Bugg, C. E. (1989). *Science*, **246**, 651–654.

McKay, E. J. (1981). *Thromb. Res.* **21**, 375–382.

Schreuder, H. A., de Boer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J. & Hol, W. G. J. (1994). *Nature Struct. Biol.* **1**, 48–54.

Skinner, R., Abrahams, J.-P., Whisstock, J. C., Lesk, A. M., Carrell, R. W. & Wardell, M. R. (1997). *J. Mol. Biol.* **266**, 601–609.

Velicov, P. G., Alexander, J. I. & Rosenberg, F. (1996). *Phys. Rev. E*, **54**, 6650–6660.

Wardell, M. R., Abrahams, J.-P., Bruce, D., Skinner, R. & Leslie, A. G. W. (1993). *J. Mol. Biol.* **234**, 1253–1258.