Crystallization within agarose gel in microgravity improves the quality of thaumatin crystals

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To prevent crystals from moving in orbit and sedimenting upon their return to earth, the model protein thaumatin was crystallized in agarose gel in the Advanced Protein Crystallization Facility during the eight-day Space Shuttle mission STS-95 (November 1998). The quality of tetragonal crystals grown in microgravity was compared with that of controls prepared in parallel in the laboratory. On the basis of their diffraction properties, microgravity crystals were more ordered than crystals grown in gel on earth (the latter being, on average, better than reference crystals obtained in solution on earth). It is concluded that protein crystallization within a gel in microgravity may yield crystals of superior quality by combining the advantages of both environments. A possible explanation for the positive effect of microgravity on protein crystallization in gels involving the better quality of the nucleus is discussed.

1. Introduction

The absence of convection and of sedimentation is a major advantage when crystallizing biological macromolecules in microgravity (*e.g.* Littke & John, 1984; DeLucas *et al.*, 1989; Snell *et al.*, 1995; McPherson, 1996; Ng *et al.*, 1996). However, turbulence in crystallization vessels, the crystal motion frequently observed in manned orbiters and settling of crystals upon return under gravity may have deleterious consequences and thus be considered as drawbacks of otherwise successful experiments. For this reason, we have addressed the question of whether immobilizing crystals in a gel may be a remedy. It was anticipated that crystals trapped in a gel would be stationary at the position where they nucleate and would reach optimal shapes and volumes in the mother liquor as they do on earth. In addition, the mechanical properties of a gel would reduce uncontrolled perturbation transmitted from outside or generated within the crystallization medium, as well as damage to crystals during transportation from the landing site to the laboratory.

Here, we describe the analysis of crystals of the model protein thaumatin grown in agarose gel in microgravity during the fifth flight of the Advanced Protein Crystallization Facility (APCF) on the eight-day Space Shuttle mission STS-95 launched on 29 October 1998. Data are compared with those of control crystals prepared in parallel on earth. Crystals grown in solution, *i.e.* in the absence of gel, in the laboratory served as a reference. This comparison is based on mosaicity deduced from Bragg reflection profiles obtained with a quasi-planar X-ray beam (Fourme *et al.*, 1995) and conventional synchrotron diffraction intensity measurements.

2. Experimental

2.1. Protein crystallization

Thaumatin (Sigma, catalogue No. T-7638, Lot 108F0299) was crystallized as tetragonal bipyramids in the presence of sodium tartrate and 0.1 M N-(2-acetamido)-2-iminodiacetic acid (ADA) adjusted to pH 6.5 with NaOH as described previously (Ng *et al.*, 1997). Crystals were prepared simultaneously at 293 K in three sets of four dialysis (DIA) reactors consisting of a precipitant chamber separated from a protein chamber by a rotatable stopcock and having a total volume of 782 µl (Bosch *et al.*, 1992). A semipermeable membrane was inserted between the chambers. The protein chambers were filled with 188 µl thaumatin solution (35 mg ml^{-1} in water) and 0.15%(w/v) low gelling point ($T_g \simeq 301$ K) agarose (So.Bi.Gel, France). Tartrate concentrations ranged from 0.50 to 0.54 M at equilibrium. All reactors were filled simultaneously: two sets with agarose gel (for crystallization in microgravity and on earth) and another without agarose gel (for solution controls on earth). The duration of the microgravity session was 8 d and after landing reactors were returned to Strasbourg on 11 November 1998 for crystal analysis. Earth controls were activated for the same time as space reactors.

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Crystallization conditions			FWHM (arcsec)		
Environment	Medium	n†	ω_1	ω_2	$Q = \omega_1/\omega_2$‡
Space	Gel	5	12.2 ± 3.0	15.0 ± 4.7	0.83 ± 0.13
Earth	Gel	4	10.6 ± 3.2	18.8 ± 6.2	0.57 ± 0.02
Earth	Solution	9	14.2 ± 4.2	25.0 ± 6.5	0.59 ± 0.15

† n, number of crystals   ‡ Displayed values with standard deviations are the mean values of the Q ratios calculated for the n crystals.

2.2. Crystallographic methods

Mosaicity was defined by full-widths at half-maximum (FWHM) of selected Bragg reflections. Reflection profiles were recorded at 293 K on beamline D25 at LURE (Orsay, France) as described by Fourme *et al.* (1995), with a new sample holder allowing a more precise positioning of the crystals (Lorber *et al.*, 1999). Reflections on the (00*l*) and (*hk*0) planes were used to measure the global misorientation (owing to lattice tilt and/or dilatation) in two perpendicular directions. FWHM ω_1 and ω_2 were measured with reflecting planes (00*l*) and (*hk*0) of crystals oriented so that their c axis was parallel or perpendicular, respectively, to the vertical plane of the incident X-ray beam. We introduce the ratio Q, defined as $Q = \omega_1/\omega_2$, to estimate the 'misorientation isotropy'.

Complete sets of X-ray diffraction data were collected at 293 K on wiggler beamline BW7B (λ = 0.8337 Å) at the EMBL-Hamburg Outstation (Germany). For each crystal, 20–60 frames at high resolution (crystal-to-detector distance, 175 or 200 mm; resolution limit, 1.1 or 1.2 Å; exposure time, 20 s per 0.5° oscillation) and 10–20 frames at low resolution (crystal-to-detector distance, 400 mm; resolution limit, 2.07 Å; exposure time 5 s per 1° oscillation) were collected on a MAR345 imaging-plate detector (MAR Research, Hamburg). Data were reduced using the *HKL* package (Otwinowski & Minor, 1997); $\langle I/\sigma(I)\rangle$ values were processed using *TRUNCATE* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) without any σ cutoff.

3. Results

Space reactors returned to the laboratory contained several immobile thaumatin crystals up to 2 mm long of bipyramidal habit with fully and equally developed faces and excellent optical properties. Earth controls performed in parallel also yielded many tetragonal crystals trapped in the gel. In reference reactors without gel, unevenly developed crystals had nucleated mainly on glass surfaces or settled on the bottom of the protein chamber. Several crystals from each set of reactors were subjected to mosaicity and diffraction-intensity measurements.

For all crystals, whatever their size and crystallization conditions, the width ω_1 of the (00*l*) reflection profiles was always smaller than the width ω_2 of the (*hk*0) reflection profiles, and $\omega_1 \leq \omega_2 \leq 2\omega_1$. A similar phenomenon was observed for lysozyme crystals (Robert *et al.*, in preparation). Mean values of ω_1 and ω_2 for 18 crystals are given in Table 1. For crystals grown either in gel or in solution, the ranges of ω_1 differed slightly but not significantly. For crystals grown in gel in space and on earth, the ranges only partially overlapped with that for crystals produced in solution on earth (which had larger ω_1 values). Mean ω_2 values were more variable. The ranges for space and earth crystals in gel overlapped less than did the corresponding ranges of ω_1. Furthermore, the ranges for crystals in gel in space and in solution on earth had almost no common values. The mean value of the Q ratio for crystals grown in gel in space (0.83) was significantly superior to that for crystals grown on earth either in gel (0.57) or in solution (0.59). However, in the latter cases standard deviations were different (Table 1). From these results, we have deduced that best crystals had the smallest ω_1 values and a Q ratio closest to 1 (which corresponds to a misorientation isotropy). Consequently, the quality of crystals prepared in gel on earth was intermediate between that of the better crystals grown in gel in space and that of less good crystals grown in solution on earth.

The statistics for diffraction data collected with a synchrotron source on nine crystals of similar size are summarized in Table 2. Independently of their origin, crystals belong to the same tetragonal space group with nearly identical unit-cell parameters. In the 1.2–20 Å resolution range, data sets of each group of three crystals are complete to $\sim$98% with a good redundancy ($\sim$5) and low R_{sym} values ($\sim$3.5%). The apparent mosaicity estimated from these oscillation data is slightly lower and more uniform for crystals from space (Table 2).

Plots of $\langle I/\sigma(I)\rangle$ as a function of resolution, displayed in Fig. 1, show that data for crystals in gel returning from space are better than, although very close to, those of crystals grown in gel on earth. Plots converge at a resolution between 1.4 and 1.3 Å and reflections are observed at 1.1 Å resolution for all crystals. $\langle I/\sigma(I)\rangle$ values for crystals grown in solution were on average 20% lower than those for crystals grown in gel as indicated by the ratio $\langle I/\sigma(I)\rangle_{gel}/\langle I/\sigma(I)\rangle_{solution}$ (Fig. 1). A density map of excellent quality was computed by molecular replacement (not shown) and an anisotropic refinement of thaumatin structure is in progress.

4. Discussion

For a century, gels have been used to grow crystals of salts (Henisch, 1988) and a decade ago they were introduced in the field of proteins (Robert & Lefaucheux, 1988). Upon cooling below its gelling temperature, an aqueous sol of the polygalactoside agarose purified from seaweed forms a hydrophilic and thermoreversible hydrogel (Guenet, 1992). This consists of a liquid phase contained inside a macroporous solid which is an entanglement of rigid chains associated *via* van der Waals interactions and hydrogen bonds, the structure of which varies with its concentration and the ionic strength (Maaloum *et al.*, 1998). When used for protein crystallization, this gel behaves as a neutral network in which convection is reduced and supersaturation evenly distributed (Vidal *et al.*, 1998). Crystals nucleate and grow inside its pores and remain stationary at their original position in the mother liquor. Owing to the loose structure and the flexibility of low-concentration [0.1–0.2%(w/v)] gels, soft protein crystals can fully develop in three dimensions and achieve near to perfect habits.

Superior diffraction intensities collected from crystals of human serum albumin prepared in agarose gel were the first evidence for a potential quality improvement compared with control crystals grown in solution (Miller *et al.*, 1992; DeLucas *et al.*, 1994). In the case of hen egg-white lysozyme, crystal mosaicity was reduced when growth had taken place in such a gel (Vidal *et al.*, 1999). For thaumatin crystals grown in gel, our results show a correlation between

Table 2
Statistics for data sets collected on space- and earth-grown thaumatin crystals.

Values in parentheses refer to the last resolution shell (1.20–1.23 Å).

Crystallization conditions	Space gel	Earth gel	Earth solution
Number of crystals	3	3	3
Apparent mosaicity† (°)	0.07, 0.08, 0.08‡	0.08, 0.08, 0.1	0.07, 0.08, 0.45
Number of observations	492938	451378	389200
Number of unique reflections	81012	81577	81838
Space group	$P4_12_12$	$P4_12_12$	$P4_12_12$
Unit-cell dimensions (Å)	$a = 58.53, c = 151.35$	$a = 58.53, c = 151.35$	$a = 58.54, c = 151.35$
Resolution range (Å)	1.2–20	1.2–20	1.2–20
Completeness (%)	97.4 (95.6)	98.2 (96.7)	98.4 (91.2)
R_{sym} (%)	3.5 (50.4)	3.4 (51.1)	3.7 (50.1)
$\langle I/\sigma(I)\rangle$	17.9 (1.91)	17.9 (1.91)	17.9 (1.91)

† The apparent mosaicity is the rocking angle (1° = 3600 arcsec) in the vertical and horizontal directions which could generate all diffraction spots seen on one still frame. It includes contributions from X-ray bandwidth and beam divergence (Otwinowski & Minor, 1997) and is consequently more than one order of magnitude larger than mosaicity measured by topography. ‡ The mosaicity of this crystals was 0.15° when it was measured a few hours later.

the small difference in misorientation existing along two perpendicular directions and the higher diffraction intensity. As for crystals of the same protein prepared in solution (Ng *et al.*, 1997), the quality was improved when nucleation and growth had occurred in microgravity. However, no direct comparison can be made between previous and present experiments because crystal sizes (0.5 mm along the *c* axis in the former and 1 mm in the present study) and analytical tools (classic synchrotron beamline D2AM at ESRF, Grenoble *versus* topographic beamline D25 at LURE, Orsay) were different. Here, the gel had immobilized crystals during their growth and protected them from mechanical shock (leading to abrasion) during the re-entry of the Shuttle into the atmosphere and during their transportation on land.

If one excepts defects generated after growth, the improvement in thaumatin crystal perfection might be the consequence of the elimination of defects of two other types: (i) nucleation defects including dislocations, subgrain boundaries or possibly twin boundaries affecting the nucleus and propagating through the whole crystal and (ii) actual growth defects including solid and liquid inclusions (which frequently generate new dislocations) or growth bands arising from the irregular supply of molecules to active surfaces as a consequence of perturbations occurring in the medium. In addition to its efficiency in preventing convection-induced perturbations, a gel might play the role of an *in situ* filter (as suggested previously by Robert & Lefaucheux, 1988), discriminating between solute molecules and large impurities such as non-specific aggregates, other nuclei and clusters
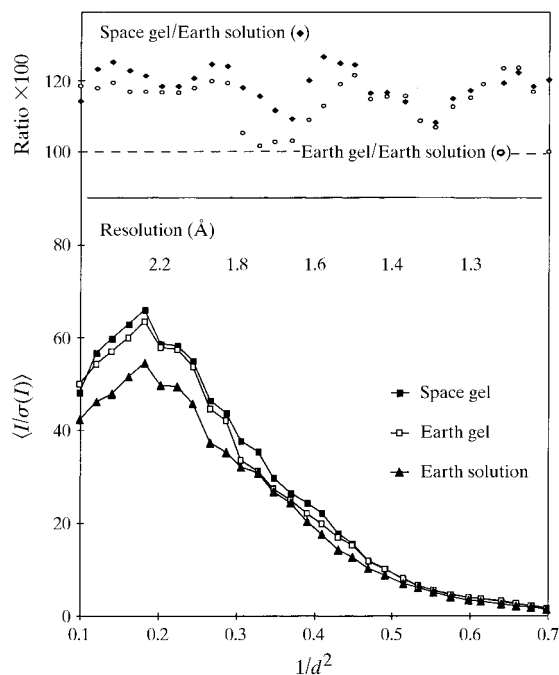

Figure 1
Graph of $\langle I/\sigma(I)\rangle$ as a function of resolution for space- and earth-grown thaumatin crystals. Average values of three crystals in agarose gel prepared in space, of three crystals grown in gel and three grown in solution on earth were used. All crystals were of similar volume, with a length of 1.0 ± 0.1 mm. $\langle I/\sigma(I)\rangle$ was computed on all reflections without any σ cutoff. Ratios of $\langle I/\sigma(I)\rangle$ of space–gel/earth–solution crystals and of earth–gel/earth–solution crystals are displayed in the top panel. In the resolution range 3.2–1.2 Å, crystals grow in gel in space are slightly better than controls grown in gel on earth. However, both gel crystals yield signal-to-noise ratios which are on average 20% higher than those of reference crystals prepared in solution on earth. Similar ratios were obtained at lower resolution (results not shown).

(Malkin *et al.*, 1996; Carter *et al.*, 1999; McPherson *et al.*, 1999). Therefore, the only effect that was anticipated was a better reproducibility of the quality of crystals grown in gel in space (and on earth) with respect to that of crystals grown in solution on earth (Vidal *et al.*, 1999; Lorber *et al.*, 1999). No significant difference in macromolecular impurities between crystal content and original thaumatin solution was detected with current analytical methods.

Since thaumatin crystals having nucleated in the gel on earth were less ordered, we conclude that their nucleation process has been perturbed by the gravity field. On the scale of the pore size (~1 μm), nucleation can be considered to occur in a gel-free volume of solution. A detailed X-ray topographic study of crystals grown on earth (Robert *et al.*, in preparation) has shown that their main defect is a misorientation generated at the seed level between parts growing in opposite directions. Since this defect was less important in crystals which had nucleated in space, the quality improvement might be a consequence of the better quality of the nuclei. The same observation has been reported for mineral crystals: contrary to earth-grown crystals, the central part of crystals having nucleated in space was strain-free and no dislocation was generated during nucleation (Robert *et al.*, 1988).

5. Conclusions

By employing an agarose gel in a microgravity environment, new insights into the mechanisms of protein crystallogenesis were achieved. Crystals of superior and more uniform crystallographic quality were obtained in this medium in microgravity compared with controls prepared under otherwise identical conditions on earth, with a 30% improvement in misorientation isotropy. Further, crystals grown in agarose gel either in microgravity or on earth were significantly better than reference crystals prepared in parallel in solution, with a diffraction signal which was 20% more intense on average. The use of the gel allowed distinction between defects generated during nucleation and those produced during growth. Comparative characterization of the crystals suggests that the nucleation process was influenced by the gravity field. This novel application of gels awaits generalization to other macromolecules.

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