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Protein Crystal Growth in the Advanced Protein Crystallization Facility on the LMS Mission: a Comparison of *Sulfolobus solfataricus* Alcohol Dehydrogenase Crystals Grown on the Ground and in Microgravity

L. Esposito,[a] F. Sica,[a] G. Sorrentino,[a] R. Berisio,[a] L. Carotenuto,[b] A. Giordano,[c] C. A. Raia,[c] M. Rossi,[c] V. S. Lamzin,[d] K. S. Wilson[d] and A. Zagari[a]*

[a]*Centro di Studio di Biocristallografia, CNR and Dipartimento di Chimica, Università di Napoli 'Federico II', Via Mezzocannone 4, 80134 Naples, Italy,* [b]*Microgravity Advanced Research & Support Center (MARS), Via Comunale Tavernola, 80144 Naples, Italy,* [c]*Istituto di Biochimica delle Proteine ed Enzimologia, CNR, Via Marconi 10 Naples, Italy, and* [d]*European Molecular Biology Laboratory (EMBL) c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany. E-mail: zagari@indigo2.dichi.unina.it*

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Abstract

Crystals of alcohol dehydrogenase from *Sulfolobus solfataricus* were grown in the Advanced Protein Crystallization Facility during the Life and Microgravity Sciences Spacelab mission on the US Space Shuttle. Large diffracting crystals were obtained by dialysis, whereas only poor-quality crystals were obtained by vapour diffusion. The quality of both the microgravity and ground-based crystals was analysed by X-ray diffraction. There was some improvement in terms of size and diffraction resolution limit for the microgravity crystals. However, the twinning observed in the Earth-grown crystals was also present for those grown in microgravity.

1. Introduction

In protein crystallography, the growth of high-quality crystals is still one of the most critical steps. Even though much progress has been made either in understanding the nucleation and growth mechanism, and in parallel in developing techniques to obtain protein crystals, the growth of single well diffracting crystals is often an unsuccessful and time-consuming process. This is the case for a protein currently under investigation in our laboratory. The protein is expressed by a hyperthermophilic archaeon, *S. solfataricus*, which thrives at a temperature of 360 K and at a pH of 3.5 in the Solfatara volcanic area near Naples (Trincone *et al.*, 1990). It is an NAD^+-dependent alcohol dehydrogenase (SsADH), which is able to function in such extreme life conditions. SsADH has been well characterized: it is an oligomeric zinc-containing enzyme consisting of four chemically identical subunits each of 37 kDa endowed with a broad substrate specificity and high stereo-selectivity (Raia *et al.*, 1996 and references therein). Moreover, SsADH has a remarkable stability to heat and protein denaturants and its activity increases with temperature up to 368 K, when denaturation is presumed to occur. For these reasons, this enzyme has a potential utilization for biotechnological applications. Because of these peculiar properties, the elucidation of its three-dimensional structure should contribute to understanding its structure–function relationships and providing information for the design of mutants with controlled and selected properties.

We obtained crystals of SsADH in the apo and the holo form complexed with NADH. Both crystal forms diffracted to better than 3 Å resolution (Pearl *et al.*, 1993). The crystals can assume a variety of habits, depending on the crystallization conditions and the growth method but the most common morphology has tail-coat shaped ends. Disappointingly, analysis of their diffraction pattern revealed that the crystals were twinned and hence not straightforwardly suitable for X-ray analysis. Over the past few years, with the aim of obtaining well diffracting single crystals, we have explored a large variety of different crystallization conditions and other techniques such as cross-seeding or growth in agarose gels. Only growth in agarose gels occasionally resulted in some untwinned crystals (Sica *et al.*, 1994).

Under these difficult circumstances, we decided to carry out microgravity crystallization experiments of SsADH, as a pronounced effect of gravity on protein crystal growth became evident from a rapidly increasing number of reports (*e.g.* Helliwell, 1995). It is known that a depletion zone is established around growing crystals. On Earth, the related density gradient induces convective flows at the crystal interface and, according to some authors, they increase the occurrence of defects, dislocations and other imperfections (Pusey *et al.*, 1988; DeLucas & Bugg, 1991; Day & McPherson, 1992). In a microgravitational environment these density-driven flows are removed, thus the depletion zone should not be disturbed favouring more ordered crystal growth (McPherson, 1993; Rosenberg *et al.*,

1996; Chayen *et al.*, 1997). Finally, protein crystals from the precipitating solutions usually used tend to sediment and thus contact foreign surfaces, for instance vessels. Such surface effects may be avoided in microgravity and this may be helpful for the growth of high-quality crystals.

However, somewhat inconsistent findings have been reported from protein and virus crystallization under reduced gravity (DeLucas *et al.*, 1994). The largest and most perfect crystals of satellite tobacco mosaic virus (Day & McPherson, 1992) as well as crystals of hen egg-white lysozyme (HEWL) of improved quality (Snell *et al.*, 1995) were grown in microgravity. In contrast, microgravity crystals of two bacterial enzymes, thermolysin and lysozyme, showed much weaker diffraction (Hilgenfeld *et al.*, 1992). Moreover, Vaney *et al.* (1996) found no differences in their crystal structure analyses whether HEWL crystals grew in space or on the ground. An explanation for the varied results may lie in the relative weight of bulk transport and interfacial kinetics of the growth of any individual protein (Vekilov *et al.*, 1996). Several authors have gone to great lengths to explain the differences observed between Earth and microgravity-grown crystals. The difficulty resides in carrying out an accurate comparison which must take into account many parameters, including the mission profile. An exhaustive interpretation for microgravity crystal growth phenomena will, have to wait for a larger number of experiments to be carried out.

The purpose of our microgravity experiments was to obtain crystals of better quality than on Earth and, in particular, to reveal whether microgravity affects twinning, which had never before been tested. We performed crystallization experiments of SsADH during the Life and Microgravity Sciences (LMS) Spacelab mission on the Space Shuttle Columbia, launched into space on June 20 and re-entering on July 7, 1996.

2. Experimental

2.1. *Crystallization*

Crystals of SsADH complexed to NADH grow up to 0.8 mm in our laboratory by vapour diffusion in hanging or sitting drops, in Linbro plates with a dilution ratio of 1:2, from solutions of 2-methyl-2,4-pentane-diol (MPD) 45–50%(*v*/*v*) and a protein concentration 5–12 mg ml^{-1}, at pH 8.4, within 2–3 weeks, at 293 K. At 277 K crystallization takes about 4 weeks producing crystals of the same quality.

2.1.1. *Pre-flight experiments.* Throughout the present work a single batch of pure and freshly prepared SsADH was used (Raia *et al.*, 1996). Pre-flight and flight crystallization experiments were carried out in 80 µl vapour diffusion (HD) and in 20 µl free-interface diffusion or dialysis (FID/DIA) reactors of the Advanced Protein Crystallization Facility (APCF) manufactured by Dornier Gmbh (Bosch *et al.*, 1992) and provided by European Space Agency. Prior to the experiment on the space shuttle, we received from Dornier one FID/DIA and three HD reactors. The usual crystallizing conditions in Linbro plates were then optimized in this hardware. Since the duration of the shuttle flight was scheduled for ~17 d, particular attention was given to the duration needed for growing sufficiently large crystals.

Firstly, to assess that the protein in the presence of MPD up to ~35%(*v*/*v*) did not lead to nuclei during the time between the reactor filling and the activation in space, equivalent protein solutions were monitored by optical microscopy for one month.

In the HD reactors a dilution ratio of 2:3 instead of the usual 1:2 was found to reduce the crystallization time to 10–14 d. During these preparatory trials the crystal size never reached a dimension >0.3 mm, whereas identical reference trials conducted in Linbro plates gave rise to larger crystals (0.5–0.8 mm) with better morphologies.

In the FID/DIA reactor initial trials were carried out either in the FID or in the DIA configuration. As reference, equivalent trials were performed in glass capillaries and in dialysis buttons. The best crystals were generated in the FID/DIA reactor with the DIA configuration and this was then used throughout the rest of the work.

2.1.2. *Microgravity experiments.* Microgravity and ground reference experiments were conducted in eight crystallization reactors [six HD (80 µl) and two FID/DIA (20 µl)] under the conditions listed in Table 1.

All reactors were filled with protein and other reagent solutions in the laboratory of Professor W. Weber in Hamburg. The space reactors were integrated into the APCF and transported to the launch site at the Kennedy Space Center (KSC), USA. The ground control reactors were kept in Hamburg at DESY. Both microgravity and ground reactors were activated ~8 h after launch and deactivated 1 d before the shuttle re-entry. The experiment duration was 15 d and 10 h. Throughout the transport/storage and the crystallization experiments the temperature was kept at 275 ± 1 and 291 ± 1 K, respectively.

2.2. *Data collection*

Four microgravity-grown crystals and six ground-grown crystals were examined using synchrotron radiation on the EMBL beamline X11 at the DORIS storage ring, DESY, Hamburg. Data were recorded on a Mar Research image-plate detector. Additional data were collected in Naples at the Biocrystallographic Center from one microgravity grown and a few ground-control crystals on a DIP 2030 image-plate detector (Nonius) mounted on a rotating anode operating at

Table 1. *Results of ground and microgravity crystallization experiments*

Reactor type	No.	Final composition‡ SsADH (mg ml^{-1})	MPD [%(*v*/*v*)]	NADH (m*M*)	Results† On ground *N*	On ground *L* (mm)	In microgravity *N*	In microgravity *L* (mm)
HD	3	11–13	48–50	1	many	0.10	many	0.10
DIA	1	10	48	1	10	0.45	5	1.0

† *N* = number of crystals, *L* = maximum length. ‡ Tris–HCl buffer, 50 m*M*, pH 8.4.

40 kV and 90 mA. All data were processed and refined using the *HKL* package (Otwinowski & Minor, 1997). The crystals belong to space group *C*2, with one dimer in the asymmetric unit. The cell parameters are a = 133.0, b = 85.7, c = 70.5 Å, β = 97°.

3. Results and discussion

3.1. *Crystal harvesting*

The crystallization experiments on SsADH were simultaneously performed on Earth and in microgravity. We ensured identical experimental conditions by using the same reagent solutions, crystallization hardware and temperatures as those used in microgravity.

1 d after landing, all APCF reactors were photographed at KSC. Fig. 1 is the original photo taken by the ESA team and shows the crystals grown in microgravity inside the DIA reactor. The crystals possess the same morphology with typical tail-coat ends as observed for Earth-grown crystals. The ground-control DIA reactor contained similar crystals. All crystals were harvested from the DIA reactors, mounted in capillaries and exposed to X-ray radiation. However, a large number of very small crystals were harvested in all HD reactors both in microgravity and on Earth. These crystals diffracted very poorly and were not used in our analysis. A summary of the microgravity and ground crystallization experiments is given in Table 1.

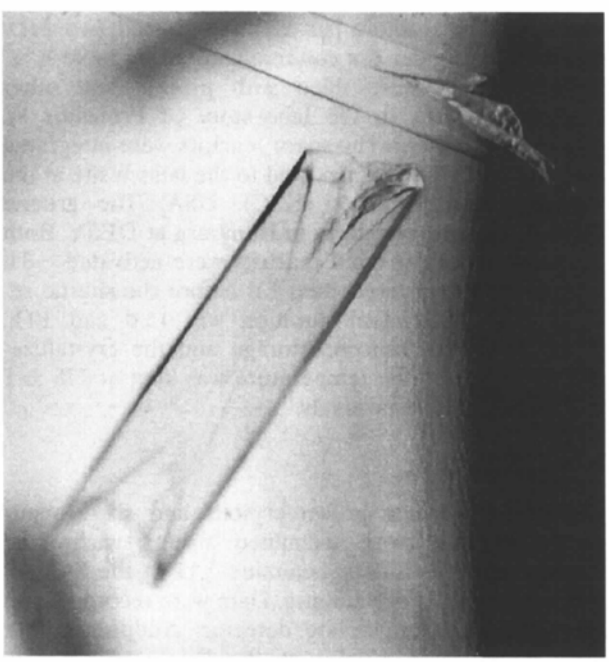

Fig. 1. Photograph taken at Kennedy Space Centre 1 d after landing. Crystals of SsADH complexed to NADH are clearly visible showing typical tail-coat shaped ends. The larger crystal has dimension 1 × 0.3 × 0.1 mm.

We obtained very different results from the HD and DIA reactors even though we started out with identical reagent solutions. This is not the first instance of such variations. For example, McPherson and co-workers obtained very different results from a protein (canavalin) and for satellite tobacco mosaic virus, during the International Microgravity Laboratory 1 mission (Day & McPherson, 1992). The best crystals of canavalin were grown by vapour diffusion, whereas the best crystals of the virus came from liquid–liquid diffusion. Chayen *et al.* (1997) described a video-camera observation of a microgravity protein crystallization in identical HD vapour-diffusion reactors: the growing crystals displayed a motion that was attributed to Marangoni effects, arising from the presence of free liquid surfaces open to vapour. Taken together, our results and those cited in the literature provide evidence for the importance of the methodology and the experimental apparatus used in protein crystallization.

3.2. *Crystal quality assessment*

The crystal quality was mainly assessed by X-ray diffraction. Firstly, we conducted a comparative analysis for microgravity-grown and Earth-control crystals at EMBL-DESY, Hamburg. Under the same operating conditions, microgravity- and Earth-grown crystals initially diffracted well, up to 1.8 and 2.5 Å resolution, respectively. Thus, the microgravity-grown crystals diffracted to a significantly higher resolution indicating an improvement in the crystal quality. Unfortunately, we were not able to collect a complete set of data to the maximum resolution, because these crystals suffered severe decay under exposure to the synchrotron beam. However, for one microgravity-grown crystal, we were able to collect complete data, although to 2.3 Å resolution. The ground-control crystals showed rapid

Table 2. *Diffraction quality of crystals on the ground and in microgravity*

Crystal growth	Crystal size (mm)	X-ray source	Resolution (Å) Limit†	Range‡	Oscillation range (°)	Completeness (%)	R_{sym}§
Microgravity	1.0 × 0.34 × 0.1	X11 Doris (λ = 0.912 Å)	1.8	18.0–2.3	1.3	89	0.08
Ground	0.43 × 0.20 × 0.1	X11 Doris (λ = 0.912 Å)	2.5	17.0–3.2	1.3	57	0.17
Microgravity	1.0 × 0.15 × 0.1	Nonius FR591 (λ = 1.542 Å)	2.6	17.0–3.0	1.1	79	0.12
Ground¶	0.45 × 0.15 × 0.1 0.40 × 0.13 × 0.1	Nonius FR591 (λ = 1.542 Å)	2.9	18.0–3.5	1.1	72	0.12

† Resolution limit to which significant data are initially observed. ‡ Resolution range where data have been recorded. § $R_{sym} = \sum(I_i - \langle I \rangle)^2 / \sum I_i^2$ where I_i is the measured intensity of an individual reflection, and $\langle I \rangle$ the mean intensity of symmetry-related measurements of this reflection. ¶ Laboratory data were merged from two crystals.

decay after a few frames, hence only partial data were collected for the best ground-control crystal. Unfortunately, analysis of the diffraction pattern revealed that microgravity crystals were twinned as previously observed for those grown on Earth.

Subsequently, we analysed one microgravity-grown crystal and a few ground-control crystals in our laboratory. We collected complete data from the microgravity crystal, albeit at a lower resolution than in Hamburg due to the lower source intensity. Earth-grown crystals again showed greater damage under exposure to conventional source X-rays. Because of the decay, only partial data sets could be recorded from single crystals and data were merged from two crystals. Establishment of cryogenic conditions will be important to extend the lifetime of the crystals in the X-ray beam. For all samples we were able to index spots on the same crystal lattice. A summary of all X-ray data is shown in Table 2. A detailed analysis of the twinning is currently in progress and our next attempt will be the exclusion of overlapping reflections.

4. Conclusions

From the findings obtained in this microgravity crystallization experiment the following conclusions may be drawn.

Firstly, the size of the SsADH microgravity crystals is larger than that of ground-control crystals, though still comparable to the best counterpart grown on Earth. This is in line with several but not all cases cited in the literature (Helliwell, 1995 and references therein; Chayen, Gordon *et al.*, 1996). Secondly, SsADH crystals grown in microgravity yielded diffraction data to a significantly higher resolution, indicating a lower extent of overall statistical disorder in microgravity-grown crystals. In addition, considering the severe decay shown by all SsADH crystals, we observed increased stability of microgravity crystals to X-rays, under the same operating conditions. Furthermore, the influence of the growth method was particularly pronounced for SsADH; this is an open question under investigation by several laboratories (Chayen, Boggon *et al.*, 1996, and references therein).

As far as twinning is concerned, this is the first experiment where twinned protein crystals have been grown in microgravity. Microgravity crystals of SsADH complexed to NADH remain twinned. This suggests further studies on other systems of which the origin of twinning is well known, *e.g.* stress, secondary nucleation, *etc.*, and may be expected to be affected under reduced gravity.

As a general conclusion, further experiments should be planned and carried out in the field of microgravity protein crystallization to produce a larger amount of data from which rational trends can be derived.

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