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Biochemical and Biophysical Research Communications 324 (2004) 1081–1086
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Protein crystal growth on board Shenzhou 3: a concerted effort improves crystal diffraction quality and facilitates structure determination

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Received 18 August 2004

Abstract

The crystallization of 16 proteins was carried out using 60 wells on board Shenzhou 3 in 2002. Although the mission was only 7 days, careful and concerted planning at all stages made it possible to obtain crystals of improved quality compared to their ground controls for some of the proteins. Significantly improved resolutions were obtained from diffracted crystals of 4 proteins. A complete data set from a space crystal of the PEP carboxykinase yielded significantly higher resolution (1.46 Å vs. 1.87 Å), *I*/sigma (22.4 vs. 15.5), and a lower average temperature factor (29.2 $Å^2$ vs. 42.9 $Å^2$) than the best ground-based control crystal. The 3-D structure of the enzyme is well improved with significant ligand density. It has been postulated that the reduced convection and absence of macromolecule sedimentation under microgravity have advantages/benefits for protein crystal growth. Improvements in experimental design for protein crystal growth in microgravity are ongoing.

Keywords: Microgravity; Protein crystallization; Phosphoenolpyruvate carboxykinase; Shenzhou 3; Crystals diffraction quality; Cytochrome-*b*5

Protein crystal growth (PCG) in microgravity has been studied for almost two decades, yet its contribution to structural biology has been contentious. The controversy regarding its evaluation is especially reflected in the Report of the American Society of Cell Biology [1] and the response by DeLucas [2]. In 2000, the National Research Council (NRC) [3] reviewed the National Aeronautics and Space Administration (NASA) supported programs of PCG in microgravity. The NRC suggested that the microgravity environment could have beneficial effects on protein crystallization. However, the NRC noted that these programs to date have actually produced little impact in the field of structural biology. Others have pointed out that the benefits of PCG in space have not been fully exploited [4]. Improved crystal quality in microgravity has been reported in several cases, including/notably:

(1) Satellite tobacco mosaic virus (STMV) crystals grown in microgravity as compared with ground-based control crystals, 1.8 Å vs. 2.3 Å resolution [5];
(2) improved data (0.98 Å vs. 1.3 Å resolution) for crystals of serine protease proteinase K grown under microgravity compared to the ground control [6];

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.166

(3) crystals from the space shuttle mission STS-95 were on average 34 times larger, with 7 times lower mosaicity and higher resolution than their earth-grown counterparts [7];
(4) higher resolution crystals, with better internal order, were reported in the case of aspartyl-tRNA synthetase, thaumatin, and other protein crystals grown under microgravity than their earth-grown counterparts [8,9].

Due to the conflicting reports, it is important to have a method to objectively evaluate the effect of microgravity on protein crystallization. About 20% of protein crystals grown in microgravity have shown a significant increase in resolution over ground-based grown crystals. An improved methodology needs to be established for PCG space missions, to significantly improve the success rate. This, in turn, is critical for evaluating/determining the utility of further microgravity studies by the crystallography community.

Here we report the PCG experiments on board the Chinese Shenzhou 3 or "Celestial Boat," hereinafter abbreviated as SZ3, a mission launched on March 25, 2002, and lasting 7 days. Although the mission was of short duration and less than 4 wells on average were allotted to each protein sample [10], improved diffraction was observed for crystals of four of the 16 proteins, in addition to improvements in crystal size and morphology for some proteins. These improvements were due, in part, to careful and concerted planning at all stages of the mission: protein preparation, quality control, transportation, crystal growth, crystal recovery, and large scale crystal freezing and data collection.

Preliminary analyses of crystal size, morphology, and resolution limits using in-house equipment were previously reported [10]. Here we present the synchrotron radiation data collection and complete analyses of all crystals from the mission, leading to a general evaluation of the overall success of the SZ3 mission.

Materials and methods

Protein crystallization. *Escherichia coli* phosphoenolpyruvate carboxykinase (PCK, ~60 kDa) was crystallized in a complex with magnesium ions, ATP, and oxalate by mixing a protein solution (12–16 mg/mL PCK in 10 mM Tris–HCl, pH 7.6, and 2 mM EDTA) one-to-one with a solution of 23% polyethylene glycol 4000, 400 mM ammonium acetate, and 200 mM sodium acetate, pH 4.5, 10 mM magnesium chloride, 4 mM oxalate, and 4 mM ATP. Ten to 20 μL drops were equilibrated against reservoirs containing 30%, 32.5%, and 35% polyethylene glycol 4000, 200 mM ammonium acetate, and 100 mM sodium acetate, pH 4.5, in the crystallization device.

Dehydroepiandrosterone sulfotransferase (DHEA-ST) is an important enzyme in steroid metabolism. It is purified as mentioned in [11]. The human enzyme has been crystallized in the presence of the substrate (DHEA). The DHEA complex crystallizes in the orthorhombic space group $P2_12_12$ with cell dimensions of $a = 74.46$, $b = 127.49$, and $c = 44.59$ Å, and data 92.9% complete to 2.15 Å resolution [12].

For the purification and crystallization of other proteins in this SZ3 mission, references can be found in [10]. Briefly for the proteins whose diffraction is analyzed in detail in this paper, a mutant of cytochrome-*b5* (Cyto-b5m) was designed to elucidate the role of Val61 in the protein and its crystallization was studied [13]. Antibacterial peptide LC1 consists of 47 amino residues, and can kill a special pathogen in some living organisms, but it is very difficult to grow its better-quality crystals (to be published). Hemorrhagin isolated from a Chinese snake venom has hemorrhagic, lethal, and caseinolytic activities [14,15].

Data collection and processing. After returning to earth, the protein crystals were retrieved from the satellite, tested in laboratory in one day after landing, and then frozen in liquid nitrogen as soon as possible. Diffraction data were collected on beamline ID-17 at the advanced photon source (APS). Radiation of wavelength 1.15 Å was used to diffract the crystals. An ADSC Quantum-210 CCD detector was used to collect 180 diffraction images in 1° increments. The diffraction data were processed using the HKL2000 program suite [16]. Software from the CCP4 package was used to determine molecular replacement solutions for the diffraction data [17]. Atomic coordinates for all of the proteins involved in this study were taken from the PDB (the PDB codes for PCK, DHEA-ST, and Cyto-b5m are 1K3C, 1J99, and 1ES1, while the PDB codes for hemorrhagin I are 1BSW (pH 7.5) and 1BUD (pH 5.0)).

Quality analysis. Comparative analysis has been performed using quality indexes popularly used for structure determination, such as resolution (d), R_{merge}, and signal-to-noise ratio. R_{merge} factor is an important index concerning different types of errors of crystal diffraction data.

Using TRUNCATE [18] from CCP4 (Collaborative Computational Project, Number 4. 1994), signal-to-noise ratio as a function of resolution was evaluated and absolute resolution limits with signal-to-noise ratio above 2.0 were confirmed.

For comparison of electron density maps using the best resolution data of PCK (for space-grown crystal and its ground counterpart), we determined its structure at 1.46 Å (space) and 1.87 Å (ground), respectively. The electron density map of ATP, computed from these data, was then analyzed and compared.

Results

Comparisons of resolution between space and ground crystals

When grown in microgravity, crystals of the four proteins, PCK, DHEA-ST, Cyto-b5m, and antibacterial peptide LC1 among the analyzed protein crystals, were all found to diffract to higher resolution compared to analogous ground-based crystals.

The most significant improvement in resolution is demonstrated in the case of PCK. The best crystals diffracted to a 1.46 Å resolution, an improvement of 0.41 Å when compared to the ground control PCK crystal.

In the case of Cyto-b5m, there was an improvement of 0.2 Å in resolution when examined with an in-house MarResearch image plate detector. Unfortunately, the other space-grown crystals of Cyto-b5m sent to Chicago were damaged in transport to the APS and were not able to be further examined.

For DHEA-ST, the data collection at APS has shown a modest improvement of 0.11 Å. In the case of Antibac peptide LC1, the space data were significantly better as compared to the ground-based crystals, but no useful diffraction information could be obtained from the ground crystals due to their small size (Table 1).

Table 1
Highest absolute resolution limits calculated for some proteins

Protein	Space-grown (Å)	Ground-grown crystal (Å)
PCK	1.46	1.87
DHA-ST	2.71	2.82
Cyto-b5m[a]	1.60	1.80
Antibac.peptide LC1	2.00[b]	–
Hemorrhagin	2.03	1.41

[a] Due to the crystals destroyed through custom, the values shown here are the diffraction limits measured by MarResearch IP detector with a low-power fixed X-ray anode.
[b] Crystals too small.

In the case of PCK and DHEA-ST, more crystals were submitted to data collection, and the crystal resolutions can be compared using a standard deviation: we have thus 1.66 ± 0.14 Å for the space crystals, as compared to 1.94 ± 0.10 Å for ground crystals in the case of PCK; 3.03 ± 0.16 Å for the space crystals and 3.15 ± 0.49 Å for ground crystals in the case of DHEA-ST. The detailed data collection statistics are listed in Table 2.

Among all the protein crystals which can be analyzed, only hemorrhagin I from snake venom yielded a better crystal on the ground-based control. Other proteins produced crystals, which were either too small or damaged due to different reasons. In this study 4 out of 16 proteins in microgravity produced crystals that diffracted to higher resolution than did the ground-based control crystals; this success rate is only slightly better than the 20% rate reported in the literature [4], even though most of the proteins involved in this mission underwent microgravity experiment for the first time and only 3–6

Table 2
Data processing summary for PCK and DHEA-ST crystals

PCK	A-4-3	A-3-4	A-3-3	A-4-2	A-5-1	B-2-3	B-1-4	B-1-3
Space group	C2							
Cell dimensions	125.28	125.08	126.77	126.01	125.11	126.41	126.65	126.43
	91.20	89.76	90.25	89.03	90.64	90.10	90.84	90.69
	46.64	46.50	46.85	46.68	46.57	46.77	46.90	46.81
	90	90	90	90	90	90	90	90
	96.04	95.97	96.21	96.10	95.84	96.30	96.42	96.33
	90	90	90	90	90	90	90	90
Observations	254,007	160,621	200,900	103,849	118,406	130,586	98,804	109,942
Unique reflections	80,669	55,657	57,841	39,945	48,838	38,846	28,164	36,807
Resolution range (Å)	1.46	1.61	1.70	1.85	1.69	1.87	2.05	1.90
Completeness (%)	89.4	83.6	99.3	90.8	84.3	90.2	85	88.8
R_{merge} (%)	4.2	3.5	6.8	4.7	5.2	6.6	8.4	8.4
Overall I/δ	22.4	27.5	12.3	16.4	14.1	15.5	16.8	11.8
Beamline	APS 17-id							
Detector	CCD ADSC Quantum-210							
Wavelength	1.15							
Number of frames	180							
Distance of detector to crystal (mm)	100	110	100	120	100	100	170	150

DHEA		A-2-4		A-1-1	B-1-2	B-1-1		
Space group	P21212							
Cell dimensions		74.99		75.51	75.09	75.11		
		133.51		133.86	130.70	132.69		
		203.19		206.58	196.78	201.36		
Observations		371,524		218,843	153,800	297,511		
Unique reflections		57,198		36,926	24,760	49,460		
Resolution range (Å)		2.70		3.10	3.50	2.80		
Completeness (%)		99.2		97.0	98.7	97.6		
R_{merge} (%)		6.0		6.4	10.3	6.2		
Beamline	APS 17-id							
Detector	CCD ADSC Quantum-210							
Wavelength	1.15							
Number of Frames	180							
Exposure time		6		10	2	10		
Distance of detector to crystal		200		160	160	160		

wells were used for each protein which is considerably less than all the other missions carried out after 1990, where generally 9–12 wells for each protein have been used [19].

Comparisons of signal-to-noise ratio $I/\sigma(I)$ and R_{merge} (Wilson Plot)

Data were compared from microgravity-grown and ground-based crystals with similar volumes. The plots of the $I/\sigma(I)$ and the R_{merge} values at different diffraction shells versus the resolution indicated significant improvement for space crystals (Fig. 1). For PCK, this is clearly seen in the comparison between the best space and ground crystals (Fig. 1A: A-4-3 vs. B-2-3), as well as in nearly all the other comparisons (Fig. 1B: A-3-4 vs. B-1-4 and other data not shown). As far as the overall $I/\sigma(I)$ is concerned (Table 2), the advantage of space crystals is also shown, e.g., this ratio reached 22.4 for the best space PCK crystal, while it was 15.5 for its ground-based counterpart. The R_{merge} for the best space-grown crystal (A-4-3) is 4.2%, which is significantly better than that (6.6%) for the best ground crystal (B-2-3). In fact, the space-grown crystals yielded higher $I/\sigma(I)$ values and smaller R_{merge} values as compared to those of the earth-grown crystals over the entire resolution range, the difference is even more pronounced at higher resolutions (Fig. 1).

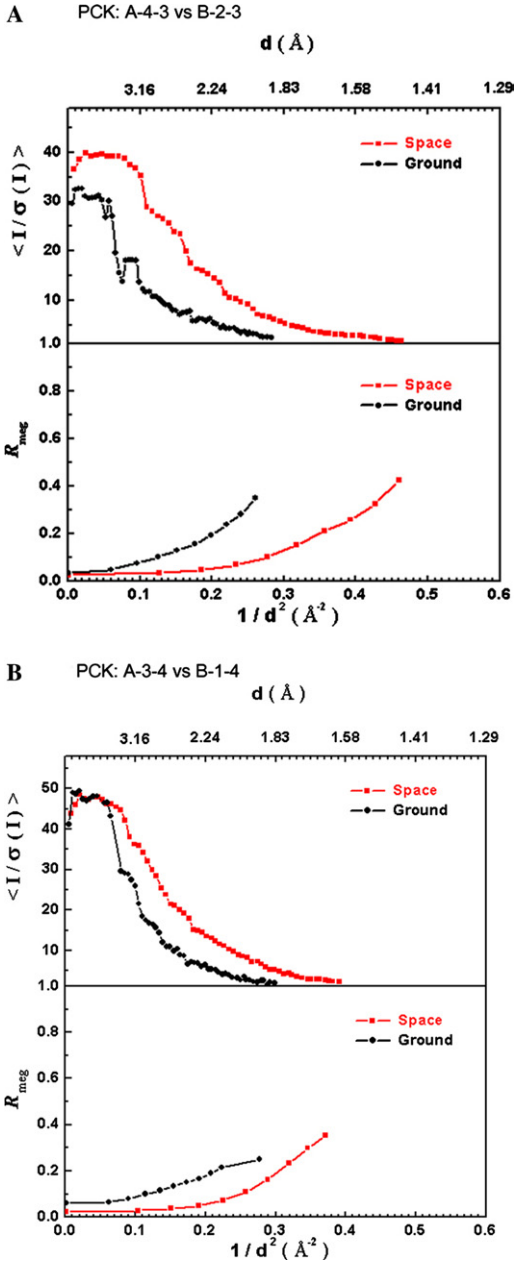

Fig. 1. Profile of the signal-to-noise ratio (I/σ (I)) and R_{merge} values versus resolution for space- and earth-grown PCK crystals.

Improvement of the electronic density around the substrate molecules in the microgravity-grown crystals

Of interest is that in the initial Fourier difference ($F_o - F_c$) map the electron density around ATP molecule is significantly better in the A-4-3 crystal than that of the B-2-3 crystal. All atoms of the ATP molecule are well defined in the initial $F_o - F_c$ map generated from the space-grown crystal (A-4-3, Fig. 2A) due to its improved quality. However, this is not the case of the ground-based crystal (B-2-3, Fig. 2B), for which the electron density corresponding to the adenine ring and ribose moieties is much weaker compared to that from the space-grown crystal. Moreover, as we can

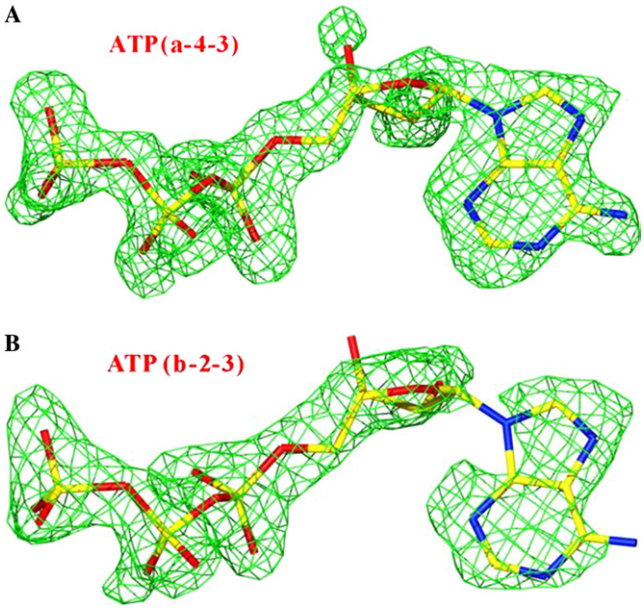

Fig. 2. Initial Fourier difference map ($F_o - F_c$.omap) for ATP molecule computed from diffraction data collected on crystals prepared either in space (A-4-3, A) or on ground (B-2-3, B). The maps are contoured at 2.0 sigma level.

see from Fig. 1B, some atoms of ATP molecule are missing in this electron density map. Thus, the improved quality of space-grown crystals may enable us to better define the positions of ligands in the targeted proteins and subsequently yield more accurate structural models, as proposed by other researchers [2,20,21].

Discussion

The SZ-3 carried out a small PCG mission accommodating 60 sample wells, with a duration of only 7 days on board the Space-Craft. The results are encouraging, as four proteins yielded crystals with improved diffraction quality, which is an important factor for structure determination. Besides some loss in the long and complete process of this PCG experiment, such as some crystals damaged in the transfer, it is clearly demonstrated that significantly improved crystals, up to more than 0.4 Å difference in diffraction and a clearly higher signal-to-noise ratio in the case of PCK, can be obtained. Together with several reports in the literature, we suggest that the PCG under microgravity remains one of the means to improve protein crystallization, which is still the bottleneck for crystallography. The methodology of crystallography resulted in most of the three-dimensional structures up to date, as shown by the coordinates deposited in the PDB bank with 85% of the total molecules determined by X-ray diffraction until May 2004 [22]. The improvement of electron density around the ATP molecule in the PCK structure also supports the idea that space-grown protein crystals are useful for structure determination and may yield more accurate three-dimensional structures [23,24,2,21,20].

A concerted effort in protein preparation, optimization of crystallization, crystal recovery, freezing of protein crystals without delay, efficient transportation of crystals, and extensive data collection with a synchrotron radiation source is very important for the evaluation of space PCG.

The advantage of PCG under microgravity likely resides in the reduction of the convection and the elimination of the density difference between protein and the solution of crystallization.

Other suggestions to obtain improved results are the following:

1. Optimizing crystallization conditions in space without any restraints as growth time period, number of samples, etc. The International Space Station could provide more opportunities for microgravity experiments. On the other hand, protein samples should be selected according to the mission condition, such as the duration.
2. Selecting suitable samples, in particular the protein crystallizations that have been shown to be sensitive to improvement with the use of microgravity, e.g., PCK and antibac.peptide LC1 may be chosen as some potential targets to be studied.
3. Improving the flight hardware, in particular increasing the number of crystallization chambers.
4. Modifying the microgravity environment in order to exploit it sufficiently, e.g., applying the Canadian Microgravity Isolation Mount (MIM) hardware to minimize the crystal movement during growth.

Acknowledgments

The authors thank the relevant researchers and technicians for making the flight hardware and preparing the experimental samples, and Dr. H. Al Dabbagh for editing of the manuscript. This study was supported by the Chinese Manned Space Flight Program and Canadian Space Agency entitled "Protein crystallization under microgravity on board the Chinese Spacecraft" for CHUL.

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