Dominique Maes, Klaas Decanniere, Ingrid Zegers, Celine Vanhee, Mike Sleutel, Ronnie Willaert, Cécile Van De Weerdt, Joseph Martial, Jean-Paul Declercq, Christine Evrard, Fermin Otalora and JuanMa Garcia-Ruiz

Protein crystallisation under microgravity conditions: What did we learn on TIM crystallisation from the Soyuz missions?

The protein Triose Phosphate Isomerase from the hyperthermophilic organism Thermotoga maritima was crystallised on board of the International Space Station in the framework of the Soyuz missions. In this paper we report on the scientific results obtained during these flights. Firstly it was shown that different crystallisation techniques and environments can result in different crystal forms for the same protein in the same crystallisation conditions, what is presumably due to a change in the rate at which supersaturation is achieved. Secondly, the X-ray quality of the crystals grown in the ISS is superior to their ground control crystals. Mimicking microgravity on ground, by adding a small amount of gel to avoid convection, also results in an improvement of X-ray quality. Nevertheless our analysis shows that the crystals obtained in this gelled ground environment are of inferior quality as compared to their space homologues. Finally we observed movement of crystals grown in the International Space Station, not only because of g-jitters but also due to residual accelerations. This has an important effect on concentration gradients of precipitants and therefore on the solubility of the protein.

Authors

Dominique Maes, Klaas Decanniere, Ingrid Zegers,
Celine Vanhee, Mike Sleutel, Ronnie Willaert
Department of Ultrastructure, Vrije Universiteit Brussel
VIB Flemish Institute of Biotechnology,
Pleinlaan 2, 1050 Brussels, Belgium

Cécile Van De Weerdt, Joseph Martial, Christine Evrard
Labo de Biologie Moléculaire et de Génie Génétique
Université de Liège, Bât. B6, Allée de la Chimie, 3, 4000 Liège, Belgium

Jean-Paul Declercq
Laboratoire de Chimie Structurale, Université Catholique de Louvain
Place Louis Pasteur 1, B-1348 Louvain

Fermin Otalora and JuanMa Garcia-Ruiz
Laboratorio de Estudios Cristalográficos, Edificio BIC Granada
Avenida de la Innovación, 1, E-18100 Armilla, Granada, Spain

Introduction

Proteins form a large and varied family of molecules, with very complex physico-chemical properties. In recent years the crystallisation of proteins has become of central importance in structural genomics projects. These projects aim at the determination of the structures of the tens of thousands of proteins for which sequences have been obtained. The availability of high resolution structures is indispensable for studying the often complex biological functions of these macromolecules, resulting in a broad variety of applications including drug design. The construction of a molecule that can exactly fit into the binding site of a protein and blocks its function can be a decisive factor in modifying a disease-related pathway.

It is clear that crystallisation is still the limiting step in the determination of detailed structures [1]. Hence there is a need for a continued research in basic aspects of protein crystallisation, as this approach leads to the development of new methods and strategies. Biological macromolecules can be crystallized by a variety of techniques, and using a wide range of reagents all at which aim to produce suitably supersaturated protein solutions. The crystallisation by counterdiffusion methods is a very efficient technique for obtaining high-quality crystals [2]. The success of this method depends on the occurrence of a supersaturation wave travelling through a protein chamber [3]. A prerequisite for the use of counterdiffusion techniques is that mass transport is controlled by diffusion alone. In solutions in which precipitants and protein diffuse, and phase separation takes place, gravity will cause density gradients and therefore sedi-

mentation and convection. This can be avoided by either working in gelled systems, working in systems of small dimensions (microfluïdics), or in the absence of gravity. For microgravity experiments, the counterdiffusion geometry has been implemented in the Advanced Protein Crystallisation Facility [4], in the Granada Crystallisation Facility [5] and in PromISS (the digital holography microscope) [6].

For our microgravity experiments we have chosen to work with proteins that can be produced in relatively large amounts, that are stable and from which the crystallisation behaviour is well characterised and reproducible. In this paper we report on the lessons we learned from the Soyuz Missions on the crystallisation of triose phosphate isomerase from *Thermotoga maritima* (TIM) [7].

Material and Methods

TIM

TIM was purified as described previously [8].

Phase diagram

The phase diagram determined as a function of ammonium sulphate and TIM concentrations was obtained using the microbatch crystallisation under oil technique [9]. The solution, consisting of the desired amount of protein and ammonium sulphate in 20 mM Tris buffer pH 8.0, 200mM NaCl , 2 mM EDTA and 0.02 % sodium azide, was first vortexed for a short time in order to obtain homogeneity. A total of 4µl was used for each drop.

Experimental setup

There were two types of reactors; the Granada Crystallisation box (GCB) [5], and the PromISS reactors. Both reactors have similar geometry, shown in Figure 1, but the PromISS reactors were developed for experiments in the digital holography microscope PromISS on the ISS and had to comply with the ESA requirements. The GCB's were put into the Granada Crystallisation Facility (GCF), essentially a big box with only a passive temperature logger [5]. In the PromISS instrument six reactors are installed and the crystallisation experiments were observed by digital holography and Mach-Zehnder interferometry, permitting the 3-dimensional reconstruction of the growing crystals and their environment and an accurate measurement of the refractive index (and therefore concentration) changes in the solution surrounding the crystal. A detailed description of the instrument is given in a previous volume of this journal [6]. For the TIM experiments the reactors were always used with a capillary counterdiffusion geometry, with the protein solution in a capillary punctured into a gel layer. The precipitant solution is layered on top of the gel, and diffuses through the gel layer into the capillary. Capillaries with diameter 0.6 mm and insertion depth dins =10 mm were used in most of the cases. All solutions contained 100 mM Tris pH 8, and 200 mM NaCl. The precipitant solution contained 4 M ammonium sulphate and the protein solution contained 25 mg/ml protein and 0.5 M ammonium sulphate at the beginning of the experiment. The gel layer contained 1 % low melting point agarose (w/v) (GIBCOBRL: Cat n° 15517-022). For each space setup a ground control was set up. Diffusion experiments on ground were carried out with and without 0.1 % agarose gel in the protein solution.

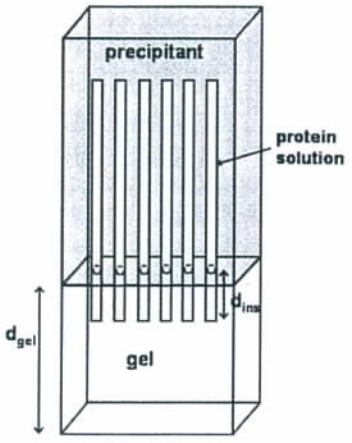

Fig. 1: The geometry of the capillary counterdiffusion experiment.

X-ray data collection

Data collection was done at the European Synchrotron Radiation Facility (ESRF) in Grenoble (France). The diffraction data were collected at 100 K on beamline BM30A (FIP) with the wavelength tuned to 1.078137 Å. All the images were recorded in the dose mode (≈ 10s in the uniform filling mode at 200 mA) on a MAR Research CCD detector placed at a distance of 150 mm from the crystal which corresponds to a maximum resolution of 2.2 Å. The oscillation angle was 0.5 degree and the slits were set to 0.3 mm. For each data set, a rotation range of 90 ° was collected. All the measurements were indexed and integrated using the program DENZO and merged with the program SCALEPACK [10].

Statistical analysis

The statistical comparison was done with the "Statistical Package for the Social Sciences" (SPSS Inc, Chicago).

Results and Discussion

Optimisation of crystallisation for the counterdiffusion technique

TIM was originally crystallised using the hanging drop vapour diffusion technique and its structure was solved to a resolution of 2.8 Å [7]. In order to determine the optimal TIM crystallisation conditions we set up a two-dimensional phase diagram exploring various TIM (1-40 mg/ml) and ammonium sulphate concentrations (0.5-2.5 M) (Figure 2). After 75 days, five zones representing different degrees of supersaturation are identified in the phase diagram: in the first (white squares), TIM remains soluble; in the second (pink), a few large TIM crystals grow in

a zone of moderate supersaturation; in the third (green), a lot of TIM crystals grow at higher salt or protein concentrations; in the fourth (yellow), needle-like crystals are present; and finally, in the fifth (blue), at high supersaturation precipitate appears. In order to obtain good crystals in the counterdiffusion setup we have to obtain conditions in our capillaries corresponding to zone 2 of the phase diagram. Moreover we have to take into account some specific aspects of the capillary counterdiffusion technique in microgravity. Firstly the nucleation induction time is much larger as compared to the vapour diffusion technique because the driving force for the mixing of protein and precipitant solution is only diffusion based. Secondly the PromISS instrument is a passive instrument. Hence the geometry has to be such that nucleation and growth occur after the reactors are installed in the ISS.

Polymorphism

All the TIM crystals grown in capillary counterdiffusion experiments were trigonal, as the crystals obtained with the hanging drop vapour diffusion technique. However the unit cell is much larger (a = 215 Å, c = 106 Å instead of a = 125 Å, c = 104.Å) [7].

The major differences between counterdiffusion and hanging drop or batch techniques are related to the rate at which supersaturation is achieved. In hanging drop and batch experiments protein and precipitant solution are mixed directly by pipetting. At the immediate contact surface between protein and precipitant solutions the supersaturation may be much higher than in the bulk solution, leading to nucleation within the boundary layer. Also, turbulent mixing of the solutions leads to highly fluctuating nucleation rates [11]. Counterdiffusion experiments on the other hand were designed to avoid turbulent mixing. The supersaturation changes gradually and continuously as protein and precipitants diffuse with a slow rise in supersaturation. A more stable typical crystal form for low supersaturation, but with a very low nucleation rate, may never get a chance in an experiment that moves fast through the zone of low supersaturation. The system might rapidly reach the part of the phase diagram where another crystal form is stable, and/or nucleates more rapidly. Thus the crystal form obtained from a particular experiment is a function of both kinetics and thermodynamics, and critically depends on the rate at which the system moves through the phase diagram.

The above polymorphism is not unique for TIM. It was also discovered for at least 2 other proteins, studied in our laboratory. A detailed study will be published elsewhere.

Crystal quality

From the diffraction data it was clear that the crystals obtained in a convection free environment are of superior quality (e.g. 2Å resolution compared to 2.8Å in a convective environment). In order to compare the diffusive environment in space with the diffusive environment on ground (ground controls with gel) we

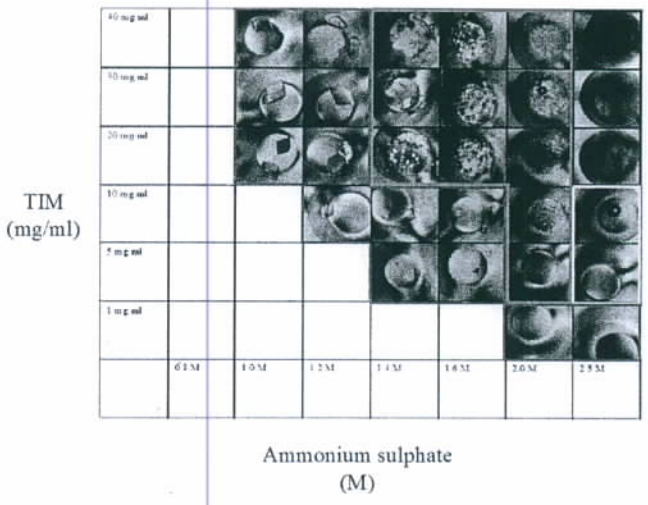

Fig. 2: The phase diagram of TIM at 20°C: microscopic observation of the microbatch experiments. The different zones are indicated with different colors.

Table 1: Comparison of the data quality parameters for agarose and microgravity-grown crystals. The mean and robust median are measures of central tendency. The standard deviation (sd) and robust interquartile range (IQR) are measures of variation.

	Ground in 0.1% agarose (16 crystals)				Space (18 crystals)			
	Mean	Median	sd	IQR	Mean	Median	sd	IQR
Mean(I/σ(I)	11.3	10.4	3.8	6.7	14.1	14.5	4.8	4.6
Wilson B (Å²)	35.7	35.3	3.8	1.6	34.7	32.6	5.5	2.8
Mosaicity (°)	0.43	0.38	0.13	0.13	0.36	0.33	0.13	0.9
Resolution (Å) at mean(I/σ(I))=10	2.97	2.79	0.64	0.72	2.75	2.59	0.53	0.31
R_{merge} (%)	8.3	7.9	1.7	6.8	6.9	6.6	2.2	1.9

compared the X-ray data quality of crystals coming from both environments. A total of 34 crystals were harvested from the space and ground experiments: 5 from the Odissea mission, 11 from the Cervantes mission, and 9 crystals from each ground controls. We picked crystals of equivalent size harvested from the upper part of the capillaries (see Figure 1), in order to have the same diffracting volume for the subsequent statistical comparisons. The equality of size was confirmed by the statistical t-test. The data sets of the different crystals were collected under identical conditions. We compared the following X-ray quality parameters: signal-to-noise ratio mean(I/σ(I)), mosaicity, B-value, resolution and R_{merge}. The SCALEPACK output [10] for all the data to 2.2 Å was used for this analysis. As most of the crystals diffracted to a higher resolution, the resolution indicated is the upperlimit of the highest data shell with a mean(I/σ(I)) equal to 10. The obtained values are reported in Table 1. Note that both the average values and the medians for all the crystal quality parameters are better for the space-grown than the ground-grown crystals.

To check if the improvement was statistically significant the robust Mann-Whitney test was used. It is a non parametric statistical test which can be used for small samples with outliers. The disadvantage of this test is that it is less powerful in spotting differences. Our comparative statistical analysis showed improved mosaicity (p-value = 0.03), B-value (p-value = 0.02) and R_{merge} (p-value = 0.01) for the space crystals at a 5% significance level. The available data showed no difference for the signal-to-noise ratio (mean(I/σ(I))) (p-value = 0.10) or the resolution (p-value = 0.17) at a 5% significance level. This does not imply that there is no difference, but that any difference present is too small to be detected with this limited data set. From the table it is clear that the interquartile range, a robust measure for the variation in the data, is larger for the ground crystals than for the space crystals for the signal-to-noise ratio, Rmerge, and resolution. The detailed statistical comparison is published elsewhere [12].

Movement of crystals

The holographic and interferometric images of the PromISS instrument show that the crystals were moving in microgravity conditions. This movement was not a mere consequence of sporadic g-jitters but points to the presence of residual accelerations. Crystals seem to move in all directions, depending on the experiment. Velocities are ≈ 10 μm /hr for 100 μm crystals, and up to 75 μm /hr for bigger crystals. This movement severely disturbs the solubility and concentration gradients and, hence, the growth behaviour. The details of this analysis were reported in an earlier study [6, 13]. We are currently studying the PromISS interferometric data in order to quantify the concentration gradients around the moving crystals.

Conclusion and further analysis

From this study it is clear that microgravity conditions and/or diffusion-limited techniques offer the possibility to grow crystals of superior X-ray quality as well as to grow crystals belonging to a different space group. The underlying reasons are not yet clear. Further analysis will concentrate on the influence of mass transport and surface growth processes on protein crystal perfection. The concepts of the concentration depletion zone [14, 15] and the impurity depletion zone [16] and their influence on crystal quality will be analysed. A detailed AFM study, describing the process of TIM crystal growth, is underway.

Acknowledgement

We thank the European Space Agency for providing the flight opportunities and for financing in the context of Prodex project C90035 and AO004. We are indebted to the Belgian Science Policy Office for financial support. The authors gratefully acknowledge the use of synchrotron beamtime at the ESRF in Grenoble and at the EMBL beamlines BW7A and BW7B at the DORIS storage ring, DESY, Hamburg.

References

1. *Chayen,N.E.:* Turning protein crystallisation from an art into a science: Current Opinion in Structural Biology vol 14:,p. 577 (2004)
2. *Garcia-Ruiz,J.M.:* Counterdiffusion methods for macromolecular crystallization. Macromolecular Crystallography, Pt C vol 368, p.130 (2003)
3. *Garcia-Ruiz,J.M., Otalora,F., Novella,M.L., Gavira,J.A., Sauter,C., Vidal,O.:* A supersaturation wave of protein crystallization. Journal of Crystal Growth vol 232, p.149 (2001)
4. *Bosch,R., Lautenschlager,P., Potthast,L., Stapelmann,J.:* Experiment Equipment for Protein Crystallization in Mu-G Facilities. Journal of Crystal Growth vol 122, p. 310 (1992)
5. *Garcia-Ruiz,J.M., Gonzalez-Ramirez,L.A., Gavira,J.A., Otalora,F.:* Granada Crystallisation Box: a new device for protein crystallisation by counter-diffusion techniques. Acta Crystallographica Section D-Biological Crystallography vol 58,p. 1638 (2002)
6. *Zegers,I., Carotenuto,L., Evrard,C., Garcia-Ruiz,J.M., De Gieter,P., Gonzales-Ramires,L., Istasse,E., Legros,J.C., Martial,J., Minetti,C., Otalora,F., Queeckers,P., Schokaert,C., Van De Weerdt,C., Willaert,R., Wyns,L., Yourassowsky,C., Dubois,F.:* Counterdiffusion protein crystallisation in microgravity and its observation with PromISS (Protein Microscope for the International Space Station). Microgravity Science and Technology, vol XVIII, Issue 3-4, p165 (2006)
7. *Maes,D., Zeelen,J.P., Thanki,N., Beaucamp,N., Alvarez,M., Thi,M.H., Backmann,J., Martial,J.A., Wyns,L., Jaenicke,R., Wierenga,R.K.:* The crystal structure of triosephosphate isomerase (TIM) from *Thermotoga maritima*: a comparative thermostability structural analysis of ten different TIM structures. Proteins vol 37, p. 441 (1999)
8. *Beaucamp,N., Hofmann,A., Kellerer,B., Jaenicke,R.:* Dissection of the gene of the bifunctional PGK-TIM fusion protein from the hyperthermophilic bacterium *Thermotoga maritima*: Design and characterization of the separate triosephosphate isomerase. Protein Science vol 6, p. 2159 (1997)
9. *Chayen,N.E., Shaw Stewart,P.D., Blow,D.M.:* Microbatch crystallization under oil — a new technique allowing many small-volume crystallization trials. Journal of Crystal Growth vol 122, p.176 (1992)
10. *Otwinowski,Z. Minor,W.:* Processing of X-ray diffraction data collected in oscillation mode. Macromolecular Crystallography, Pt A vol 276, p. 307 (1997)
11. *Chernov,A.A.:* Protein crystals and their growth. J.Struct.Biol.vol 142, p. 3 (2003)
12. *Evrard,C., Maes,D., Zegers,I., Declercq,J.P., Vanhee,C., Martial,J., Wyns,L., Van De Weerdt,C.:* TIM crystals grown by capillary counter-dif-

fusion: Statistical evidence of quality improvement in microgravity. Crystal Growth and Design vol. 7, no. 11, p. 2161-2166 (2007)
13. *Otalora,F., Novella,M.L., Gavira,J.A., Thomas,B.R., Garcia Ruiz,J.M.:* Experimental evidence for the stability of the depletion zone around a growing protein crystal under microgravity. Acta Crystallogr.D.Biol.Crystallogr. vol 57, p. 412 (2001)
14. *Mcpherson,A., Malkin,A.J., Kuznetsov,Y.G., Koszelak,S., Wells,M., Jenkins,G., Howard,J., Lawson,G.:* The effects of microgravity on protein crystallization: evidence for concentration gradients around growing crystals. Journal of Crystal Growth, 196:572-586.
15. *Vekilov,P.G., Chernov,A.A.* (2002): The physics of protein crystallization. Solid State Physics: Advances in Research and Applications vol 57, p. 1 (1999)
16. *Chernov,A.A.:* Crystal growth and crystallography. Acta Crystallogr.A vol 54, p. 859 (1998)