Crystallization of the collagen-like polypeptide $(PPG)_{10}$ aboard the International Space Station. 2. Comparison of crystal quality by X-ray diffraction

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Crystals of the collagen-like polypeptide $(PPG)_{10}$ were obtained within the Advanced Protein Crystallization Facility on board the International Space Station, during the STS-105/STS-108 mission. The duration of this mission was such to ensure that the crystallization process had reached its end. Crystals were grown both in the presence and in the absence of agarose gel, to compare the quality of the crystals obtained from these different environments. As a result, crystals grown in the absence of agarose on Earth as well as in microgravity showed X-ray diffraction up to 1.15 Å. The intensity/sigma ratio was slightly higher for microgravity grown crystals. Crystals grown in agarose gel, both in microgravity and on ground, showed a comparable diffraction power, with a resolution limit of 1.45 Å.

Keywords: **collagen; triple helix; ISS; microgravity; APCF; X-ray diffraction**

1. Introduction

Collagen is the most abundant protein in animals. It readily forms fibres, which give mechanical support to skin, tendons and bones. Although relevant progresses have recently been achieved (Orgel *et al*., 2001), the fibrous nature of collagen hinders an accurate study of its typical triple helical structural motif. In this context, the use of triple helical polypeptide collagen models has recently provided a great contribution in understanding the structural and thermodynamic properties of collagen (Persikov *et al*., 2001; Berisio *et al*., 2002a; Jenkins & Raines, 2002). Among the collagen-like polypeptides, $(Pro\text{-}Pro\text{-}Gly)_{10}$, hereafter named as $(PPG)_{10}$, certainly is the first and most widely investigated (Kramer *et al.*, 1998; Nagarajan *et al.*, 1998; Vitagliano *et al.*, 2001a; Berisio *et al.*, 2002b) since X and Y positions of the collagen consensus X-Y-Gly sequence are frequently occupied by iminoacids. This molecule has a peculiar rod-like shape and therefore, it is also an interesting case from the crystallogenetic point of view, since most of the crystallogenetic information available from the literature refers to globular proteins (McPherson, 1999). It is also worth mentioning that detailed structural studies on $(PPG)_{10}$ were initially precluded by the poor quality of the polypeptide crystals grown on Earth. Indeed, only average structures of $(PPG)_{10}$ could be derived (Kramer *et al.*, 1998; Nagarajan *et al.*, 1998).

A long-term project ongoing in our laboratory aims at studying the effect of microgravity on protein crystal growth, a still poorly understood process (Kundrot *et al.* 2001). Crystal growth of $(PPG)_{10}$ in microgravity conditions was carried out for the first time during the US space shuttle STS-95 mission (October 1998). This microgravity experiment provided larger and significantly better diffracting crystals (Berisio *et al*., 2000). These crystals X-ray data at 1.3 Å resolution provided a very detailed description of the triple helical structure of $(PPG)_{10}$ (Berisio *et al*., 2002b). In addition, the structural features emerged from this study, together with statistical structural analyses, allowed the formulation of a new mechanism which explains the effect of prolyl-hydroxylation on collagen triple helix stability (Vitagliano *et al.*, 2001a; 2001b).

Experiments carried out during shuttle flights, are restrained by the short duration of the missions themselves. Indeed, during the STS-95 mission a low gravity environment was accessible for only 8 days. Despite the efforts made to fit the crystal growth conditions to this requirement, the analysis of the CCD video images recorded aboard the space shuttle revealed that, for 5 out of 10 monitored crystals, the crystallization process would not terminate at the end of the mission (Carotenuto *et al*., 2001).

The outcome of the International Space Station (ISS) opened up new perspectives; the ISS, in fact, provides a unique low gravity environment that allows experimentation on a monthly scale (Stapelmann *et al.*, 2001). Since many space agencies promote protein crystallization experiments and provide *ad hoc* built-in facilities, we were able to perform a much longer experiment, over 3 months, on the ISS. As for the STS-95 mission, this novel $(PPG)_{10}$ crystallization process was monitored by a CCD camera (Vergara et al., 2002). To take advantage of such a long experiment aboard the ISS, microgravity crystallizations were carried out in optimised conditions, set up during pre-flight experiments. We also preliminary investigated the effect of agarose gel, successfully used for other protein systems (Lorber *et al.*, 1999; Dong *et al.*, 1999), on $(PPG)_{10}$ crystal growth both in microgravity and on ground. The results of the pre-flight as well as of the microgravity experiments are here described and discussed in terms of crystal morphology and X-ray diffraction power.

2. Materials and methods

All crystallization trials were carried out in the Advanced Crystallization Facility (APCF) reactors provided by the European Space Agency (ESA) (Bosch *et al.*, 1992; Snell *et al.*, 1996). Details regarding the reactors and the reagents used in these experiments are reported in Vergara *et al.* (2002).

2.1. Solution preparations and pre-flight crystallizations

The crystallization conditions used in the STS-95 mission (Berisio *et al.*, 2000) were used as starting conditions for the pre-flight crystallization setups. In particular, the concentration of all of the crystallization components was systematically varied in order to optimise the crystallization process, using the dialysis technique. $(PPG)_{10}$ was dissolved in a solution containing acetic acid (HAc, 1%v/v) and polyethylene glycol 400 (PEG400). Sodium acetate (NaAc) was used as a precipitant agent. For the optimisation, the final concentration of NaAc was varied in the range 0.10-0.28 M and that of PEG 400 in the range 5-15% v/v. Crystallization experiments in the presence of agarose gel were optimised also by varying agarose concentration in the range 0-0.3% w/v. The polypeptide was directly added to the agarose solution and, subsequently, the gelification process was carried out at 55 °C (one step gelification procedure) (Robert *et al.*, 1999). Indeed, although the melting temperature of $(PPG)_{10}$ is estimated to be 41 (±1) °C, its trimerisation equilibrium is fully reversible (Holmgren *et al.*, 1999).

2.2. Microgravity and ground control crystallization experiments

Microgravity experiments were carried out in four reactors, integrated in the APCF under the conditions reported in Table 1. Two different types of reactor, named as 1-block and 2-block reactors, were used with the dialysis mode. The protein chambers had a volume of 55 and 67 µl for 1-block and 2-block reactors, respectively. The precipitant was added to the reservoir, 144 µl and 278 µl for 1-block and 2-block reactors, respectively, and to the plug channel, 85 µl and 99 µl for 1-block and 2-block reactors, respectively, (see also Vergara *et al.* (2002) for additional details). For reactors n. 410 and n. 616, the protein chambers were filled with 5 mg/ml $(PPG)_{10}$ at room temperature. In the two other reactors (codes 403 and 612), agarose gel 0.2 % w/v was also added to the 5 mg/ml $(PPG)_{10}$ (Table 1). Identical crystallization conditions were used in microgavity and ground control reactors, that were filled simultaneously by using the same reagent solutions. The APCF was transported to and from the ISS by STS-105 and STS-108 flights, respectively.

Table 1

Crystallization conditions in APCF reactors

Reactor type	No.	Final (initial) composition				No. Crystals tested	Resolution limit (Å)
		pH (final)	Sodium acetate (M)	Acetic acid (%v/v)	Agarose (%w/v)		
Microgravity							
1-block	403	5.3	0.18 (0.23)	0.23 (1.00)	0.2 (0.2)	2	1.45
1-block	410	5.3	0.18 (0.23)	0.23 (1.00)	0.0 (0.0)	5	1.15
2-blocks	612	5.5	0.20 (0.23)	0.17 (1.00)	0.2 (0.2)	1	1.45
2-blocks	616	5.5	0.20 (0.23)	0.17 (1.00)	0.0 (0.0)		
Earth							
1-block	607	5.3	0.18 (0.23)	0.23 (1.00)	0.2 (0.2)	2	1.45
1-block	405*		(0.23)	(1.00)	(0.0)		
2-blocks	602	5.5	0.20 (0.23)	0.17 (1.00)	0.2 (0.2)	1	1.45
2-blocks	603	5.5	0.20 (0.23)	0.17 (1.00)	0.0 (0.0)	5	1.15

$(PPG)_{10}$ initial concentration 5 mg/ml; PEG 400 was set at constant concentration of 10 %(v/v).
* Reactor 405 dried out

2.3. Data collection and processing

A total of 16 crystals were tested to assess their highest resolution limits. In particular, 5 microgravity-grown crystals obtained in the absence of agarose (reactor code 410) and 5 from the ground reactor (code 603) were X-ray exposed. Furthermore, tests for the assessment of the highest resolution limit of agarose gel grown crystals were carried out on 3 crystals obtained in the microgravity environment (reactors 403 and 612) and on 3 crystals obtained on ground (reactors 602 and 607).

Complete sets of X-ray diffraction data were collected at Elettra (Trieste, Italy). Since all data were registered at 20 °C, a 160 mm diameter CCD detector was preferred to a 345 mm diameter Mar Research detector to speed up data collection, thus limiting crystal decay. However, due to the very short c* axis, data were registered at the resolution range of 20.0-2.15 Å. Identical data collection strategies were used for crystals grown in microgravity and on ground. In particular two datasets were collected *per* crystal in high (5.0 - 2.15 Å) and low resolution (20.0 - 3.0 Å) ranges. The two crystals used for data collection displayed a high resolution diffraction pattern similar to the other crystals that were tested. The distance of the crystal from the detector and the rotation angle $\Delta\varphi$ were 160 mm and 0.6° for high-resolution and 235 mm and 1° for low-resolution data collections, respectively. Data collection statistics for two crystals, on which nearly-complete datasets were recorded, are reported in Table 2.

Table 2

Data processing statistics

	Microgravity	Ground
Space group	$P2_12_12_1$	$P2_12_12_1$
Wavelength (Å)	1.00	1.00
Unit cell parameters (Å)		
a	26.34	26.34
b	26.88	26.90
c	182.24	182.40
Resolution range (Å)	20-2.15	20-2.15
Mosaicity (°)	0.7	0.7
Number of reflections	27452	33334
Redundancy	4.7	5.0
Completeness (%)	77.0	87.6
Last shell	71.5	68.7
Rmerge (%)	5.9	3.7
Last shell	4.8	7.4

Statistics are for two representative data sets collected on microgravity- and on ground-grown crystals using the same experimental data collection parameters.

3. Results and discussion

3.1. Pre-flight experiments

The method of dialysis was used in all trials, since it previously provided good results both on ground and in microgravity (Berisio *et al.*, 2000). The solubility of $(PPG)_{10}$ strongly depends on the pH. Usually $(PPG)_{10}$ is dissolved in acetic acid. Nuclei form and grow as pH increases by diffusing sodium acetate. Therefore, the concentration of NaAc along with the concentration of PEG400 and agarose gel, were varied systematically, in order to change the supersaturation level.

In the case of crystals grown in the presence of agarose, this was adjusted to obtain several large crystals in each reactor. Indeed, we have observed, in accordance with previous studies (Thiessen, 1994; Vidal *et al.*, 1998), that the number of crystals monotonically increases with agarose concentration. Crystals grown in agarose gel initially present a well-shaped morphology, although their surfaces become wrinkled within a month.

Crystallization conditions (Table 1) that produced best morphology crystals were finally adopted both in microgravity and on Earth control experiments.

3.2. Microgravity experiments

In the 4 month mission, from August 11st to December 17th 2001, the experiment lasted 3 months and 20 days. Crystals were grown in all of the four flown reactors that were photographed after the flight's return. Once the reactors were recovered in our laboratory they were immediately inspected; subsequently, crystals were harvested and analysed by X-ray diffraction.

3.2.1. Crystallization in the absence of agarose gel. Reactor 410, prepared in the absence of agarose, contained few crystals with a length up to 0.30-0.35 mm. A representative picture is shown in Fig. 1. The other microgravity agarose-free reactor (n. 616) contained a larger number of smaller crystals (maximum length of about 0.2 mm), though some of them were damaged. Since STS-95 and pre-flight experiments showed $(PPG)_{10}$ crystals grown in agarose-free conditions were stable for several months, this crystal damage may be attributed either to the accelerations occurred during the re-enter or to the motions of the crystals in the microgravity reactors, motions which, in some cases, ended in crashes among crystals, as monitored by the CCD video (Vergara *et al.*, 2002). As far as the ground controls are concerned, reactor 603 contained crystals not bigger than 0.2 mm whereas reactor 405 dried out.

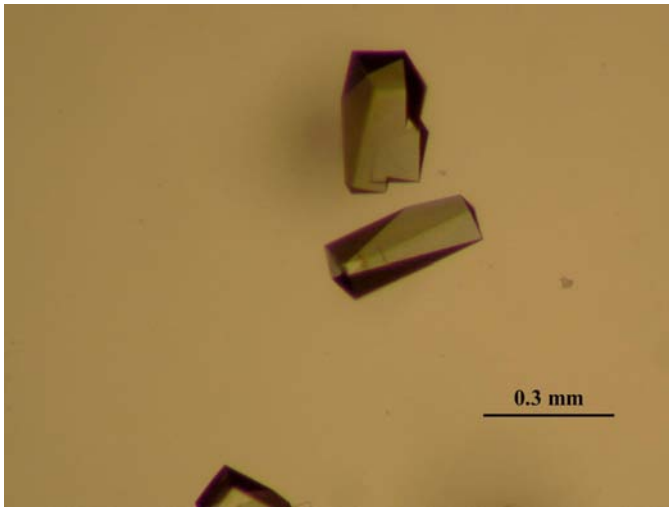

Figure 1

Photo of selected crystals in reactor n. 410 containing a gel-free solution. The photo was taken at Kennedy Space Center, one day after landing.

3.2.2. Crystallization in the presence of agarose gel. Crystals grown in agarose gel in microgravity and in the Earth control reactors were of comparable size (about 0.2 mm on their longest length). Furthermore, a high degree of surface roughness, observed on Earth after about one month, also occurs for microgravity grown crystals. The analysis of the video images has evidenced that crystals become wrinkled after about 25 days. Fig. 2 shows the surface roughness of crystals grown in reactor 612.

3.3. Comparison of the X-ray diffraction power

Ten crystals were tested for the assessment of their highest resolution limits. The analysis of the diffraction images from 10 crystals (5 microgravity- and 5 Earth-grown), obtained in the absence of agarose, has evidenced the presence of reflections at 1.15 Å resolution for crystals grown both in microgravity and on Earth. A diffraction image from a microgravity grown crystal is shown in Fig.3.

Diffraction data were collected on crystals grown either in the absence or in the presence of agarose. In order to make a very accurate comparison of the quality of the crystals grown in different environments we decided to collect complete datasets, each dataset from one single crystal, at room temperature. The flash-freezing produces a shock that is never exactly reproducible and that may increase the crystal mosaicity. Therefore it should be avoided, whenever possible, when comparing crystals. Although the crystals

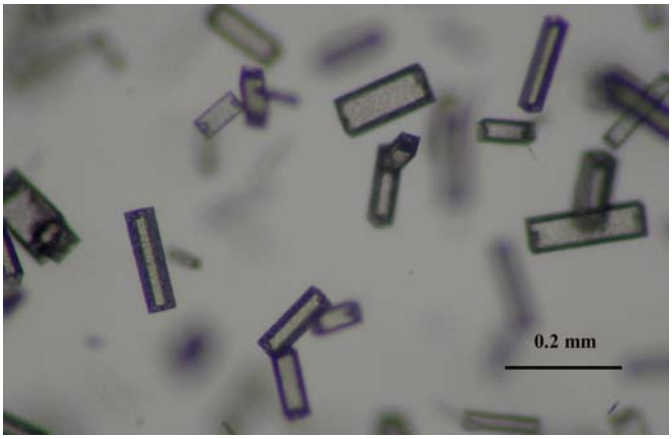

Figure 2

Crystals grown in microgravity, in reactor n. 612 containing agarose gel.

diffract at higher resolution, data were actually collected up to 2.15 Å on a fast CCD detector. This choice allowed to obtain a good spatial resolution of the intensity peaks along the c* direction, as the c axis measures 182 Å and a collection of a complete dataset without appreciable crystal decay. It is worth mentioning that high resolution (1.3 Å) diffraction data collection, which required as many as 5 crystals, and high resolution structural studies have already been reported for $(PPG)_{10}$ (Berisio *et al.*, 2000; 2002b). Nearly complete datasets were collected on one microgravity- and one Earth-grown crystal. A summary of the statistics of data processing is reported in Table 2.

The diffraction pattern of $(PPG)_{10}$ is very peculiar, since it is composed of very strong and of extremely weak reflections (Berisio *et al.*, 2000). As previously described, the indexing of only one part of the hkl reflections (those with index l = 9n) leads to a unit cell with the c axis equal to a ninth of the real axis (subcell approximation) (Berisio *et al.*, 2000). Due to the uneven distribution of the reflection intensities, they were classified in 9 classes depending on the l index, for appropriate comparisons (Table 3). In accordance with the results of the previous STS-95 mission, reflections with l = 9n were the strongest, followed by those with l = 9n+2 and l = 9n+4. For each of the reflection classes a limit resolution range was calculated as the last resolution shell containing at least 50% of the reflections having $I/\sigma(I)>3$. As shown in Table 3, reflections with l = 9n and with l = 9n+2 present $I/\sigma(I)>3$ in the whole resolution range for crystals both grown in microgravity and on Earth. By contrast, the remaining reflection classes show diffraction up to sensibly lower resolutions, slightly higher in the case of microgravity grown crystals. Consistently, reflections belonging to the highest resolution shell are about 15 % more intense in microgravity grown crystals (Table 3). On the other hand, crystals grown in the two different environments present comparable mosaicities (about 0.7°), as estimated by using the HKL package (Otwinowski & Minor, 1997).

The quality of X-ray data obtained from crystals grown in the presence of agarose gel, both in microgravity and on Earth, was lower than in its absence. Indeed, data could be processed only with the subcell approximation (Berisio *et al.*, 2000) and a very high mosaicity (about 1.1°) was estimated by using the HKL package (Otwinowski & Minor, 1997). Therefore, as already indicated by the roughness of the crystals surfaces (Fig. 2c), the lower quality of the X-ray data, at 1.45 Å, shows that crystal ageing occurs also in microgravity.

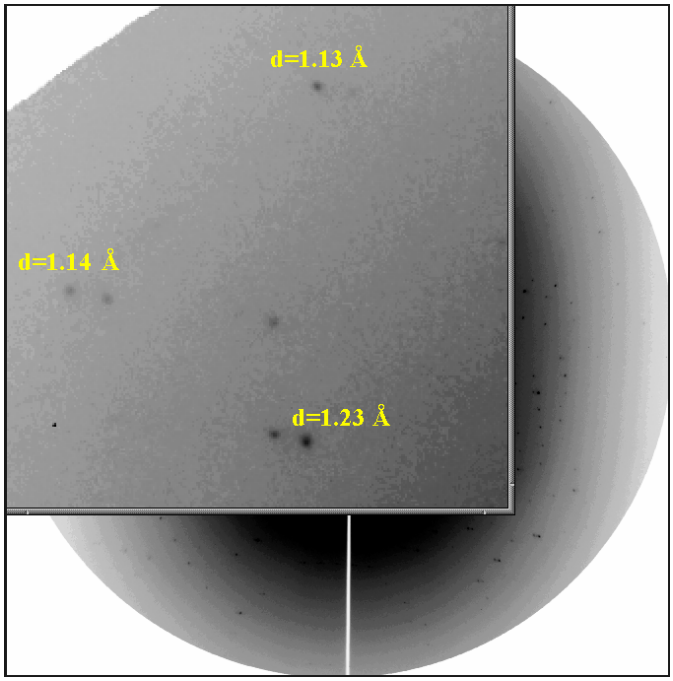

Figure 3

Diffraction image from a microgravity-grown crystal showing its highest resolution limit. The d spacing is also shown for some of the reflections. The wavelength of the X-rays used to collect this rotational image ($\Delta\phi$ = 0.6°) was 1.000 Å. The crystal to detector distance was set to 60 mm.

Table 3

Average I/σ(I) values and resolution limits for the 9 reflection classes.

l		9n	9n+1	9n+2	9n+3	9n+4	9n+5	9n+6	9n+7	9n+8
Micro-gravity	<I/σ (I)>	38.5 (39.5)	7.1 (11.0)	30.6 (33.6)	1.4 (7.8)	13.0 (17.1)	3.8 (9.4)	4.6 (10.8)	6.5 (11.1)	4.3 (9.1)
	[a] Limit resolution range (Å)	2.19-2.15	2.23-2.19	2.19-2.15	4.61-4.04	2.19-2.15	3.41-3.21	3.41-3.21	2.48-2.42	3.21-3.05
	<I/σ(I)> in the last resolution shell (2.19-2.15 Å)	34.7	2.9	17.3	0.2	4.7	1.1	0.6	2.9	0.7
Ground	<I/σ (I)>	48.0 (50.9)	3.5 (8.3)	32.2 (36.5)	1.0 (7.9)	10.9 (17.0)	2.8 (10.1)	3.0 (9.6)	4.9 (11.6)	1.9 (7.3)
	[a] Limit resolution range (Å)	2.19-2.15	3.41-3.21	2.19-2.15	4.61-4.04	2.32-2.27	4.04-3.67	3.67-3.41	3.41-3.21	4.04-3.67
	<I/σ(I)> in the last resolution shell (2.19-2.15 Å)	30.6	0.7	13.5	0.1	2.3	0.9	0.0	2.5	0.7

[a] The highest resolution shell with 50% of the reflections having <I/σ(I)> greater than 3. Values in parentheses are calculated for reflections having <I/σ(I)> greater than 3.

4. Conclusions

$(PPG)_{10}$ crystal structure has been studied independently in various laboratories. The best resolution achieved using crystals grown on ground was 1.7 Å (Kramer *et al.*, 1998; Nagarajan *et al.*, 1998). Crystals grown in microgravity during the STS-95 mission showed a significantly improved resolution up to 1.2 Å (Berisio *et al.*, 2000). A similar high resolution limit (1.15 Å) was also achieved by crystals grown in microgravity aboard the ISS (flights STS-105/108). Although counterpart crystals grown on Earth achieved a comparable resolution, they showed a slightly lower intensity/sigma ratio. Similar results have been obtained for collagenase crystals (Broutin-L'Hermite *et al.*, 2000).

The similar quality of the diffraction patterns from crystals grown in microgravity and on Earth may be ascribed to various factors such as the new crystallization conditions, the crystal motions, and the relative weight of the kinetic over transport process.

The long and systematic search for optimal conditions resulted very helpful to grow, in the absence of agarose, good quality crystals also on Earth. Indeed, differently from the STS-95 mission, in which the low gravity environment was kept only for 8 days, the experiment on the ISS was long enough to use optimised crystallization conditions and to ensure the termination of the crystallization process both on ground and in microgravity. In particular a lower initial supersaturation level was used, by decreasing the concentration of the precipitant NaAc. Under these conditions, the nuclei induction time was estimated in about 100 hours and the whole crystal growth process lasted 17-18 days. Altogether, these characteristics may have favoured a more ordered crystal formation and growth .

It should be considered that all space platforms provide a low gravity environment that suffers from residual accelerations and g-jitters, generated mostly by orbiter manoeuvres. As a result, crystal motions were observed on a number of space missions and as well as during the $(PPG)_{10}$ crystal growth. Indeed slow and coherent motions which might have reduced the benefits of microgravity were observed aboard the ISS (Vergara *et al.*, 2002).

The crystal growth process results from a balance of the protein flow towards the crystal face and the rate of incorporation into the crystal lattice. Usually, protein crystals grow in a mixed regime and it is not straightforward to know to relative weight of the two contributions. Further studies are planned, both in microgravity and on Earth, to better understand the steps of the crystal growth process of $(PPG)_{10}$.

Finally, we have also shown that well shaped crystals of $(PPG)_{10}$ can be obtained in gel both in microgravity and on ground. However, a crystal surface detriment and a lower quality of the diffraction pattern provided evidence that crystals suffered from ageing in both environments. Ground experiments have shown that these ageing effects occur also in sterilised media. Therefore, further investigations are necessary to prevent the ageing of the $(PPG)_{10}$ crystals in agarose containing media.

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