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CCD video observation of microgravity crystallization: apocrustacyanin C_1

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Abstract

Apocrustacyanin C_1 has been crystallized in the vapour-diffusion apparatus of ESA's Advanced Protein Crystallization Facility (APCF) on-board the NASA space shuttle STS-65 International Microgravity Laboratory-2 (IML-2) mission. Crystal growth was monitored by black and white CCD observation at time intervals throughout the experiment. The resulting crystals displayed a motion within the hanging drop that is attributed to Marangoni convection effects. The images also show a "halo" effect around the growing crystals which can be attributed to the presence of depletion zones i.e. solution regions which are depleted of this coloured protein.

1. Introduction

Microgravity has been used as a growth medium to prevent sedimentation and reduce buoyancy forces within crystallization media. Crystals grown in microgravity have been shown to produce enhanced signal to noise X-ray diffraction over their corresponding earth counterparts [1]. This is advantageous in collection of weak high resolution data. Several methods of microgravity crystallization are available at present; vapour diffusion, liquid–liquid diffusion, dialysis and batch. In this study the microgravity crystallization of apocrustacyanin C_1 by vapour diffusion has been monitored by CCD observation. The X-ray diffraction analysis on the resulting crystals is described elsewhere [2].

The vapour-diffusion apparatus consists of a drop containing the protein solution which is separated by an air space from a reservoir containing a concentrated precipitant solution. Water is transferred from the protein solution to the precipitant solution via vapour-diffusion thereby increasing the supersaturation in the protein solution and initiating crystallization. The transfer of protein from solution to the growing crystals results in a depleted region of protein (in comparison to the rest of the solution) around the growing crystal [3]. The associated density gradients, due to the depleted region, result in buoyancy forces and initiate convection which can be detrimental to the growing crystal [4]. Microgravity greatly reduces these buoyancy forces allowing, it is thought [5,6], a more stable depletion layer to form

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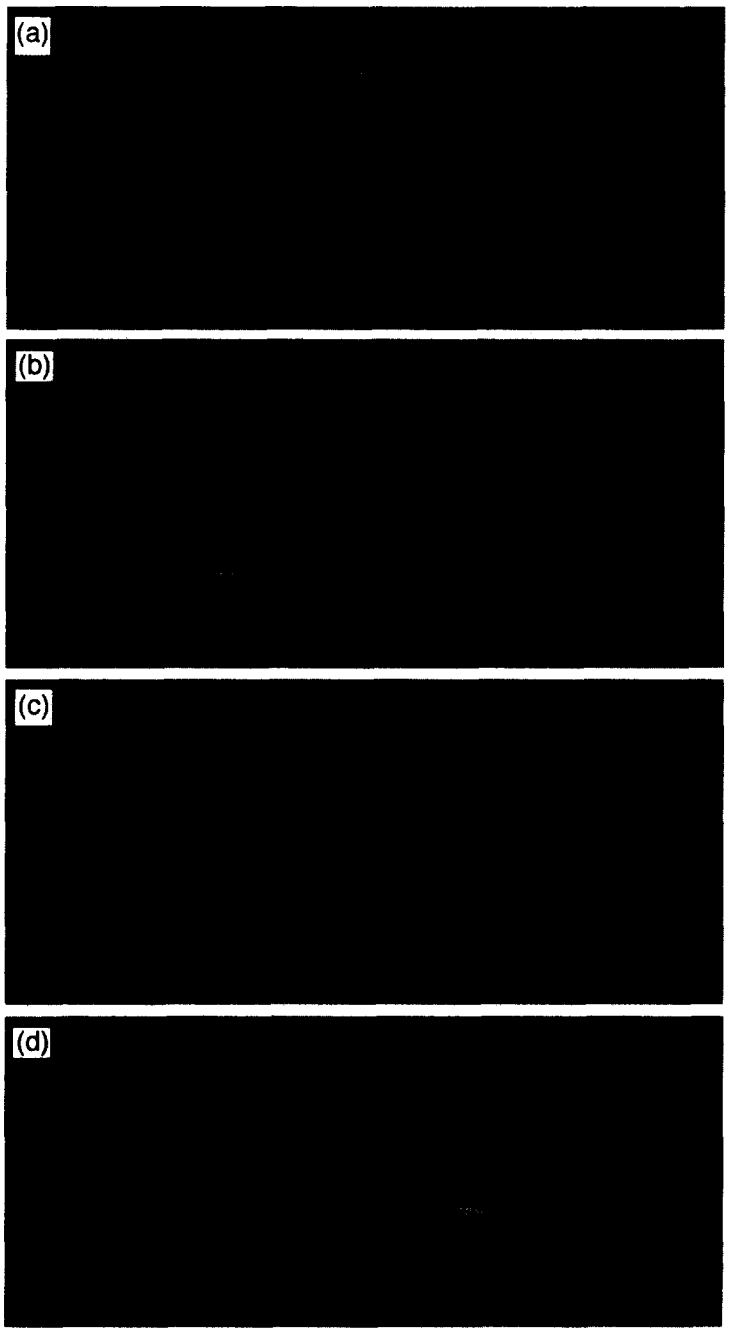

Fig. 1. CCD video images of the apocrustacyanin C_1 protein crystal growth drop taken at times of (a) 46:32 h, (b) 54:14 h, (c) 62:05 h, (d) 69:55 h, (e) 77:44 h, (f) 85:30 h, (g) 93:20 h and (h) 101:10 h. The image is oriented such that the drop appears as a hanging drop with three needle shaped crystals visible. In (a) the crystals are pointed out, surrounding them is an oval shaped "halo" or depletion zone effect. The field of view shown is 7.9 mm in the horizontal direction by 2.7 mm in the vertical direction. A pattern of movement is shown in (a)–(d) which then repeats itself in (e)–(h). The arrows highlight crystals which have changed their position.

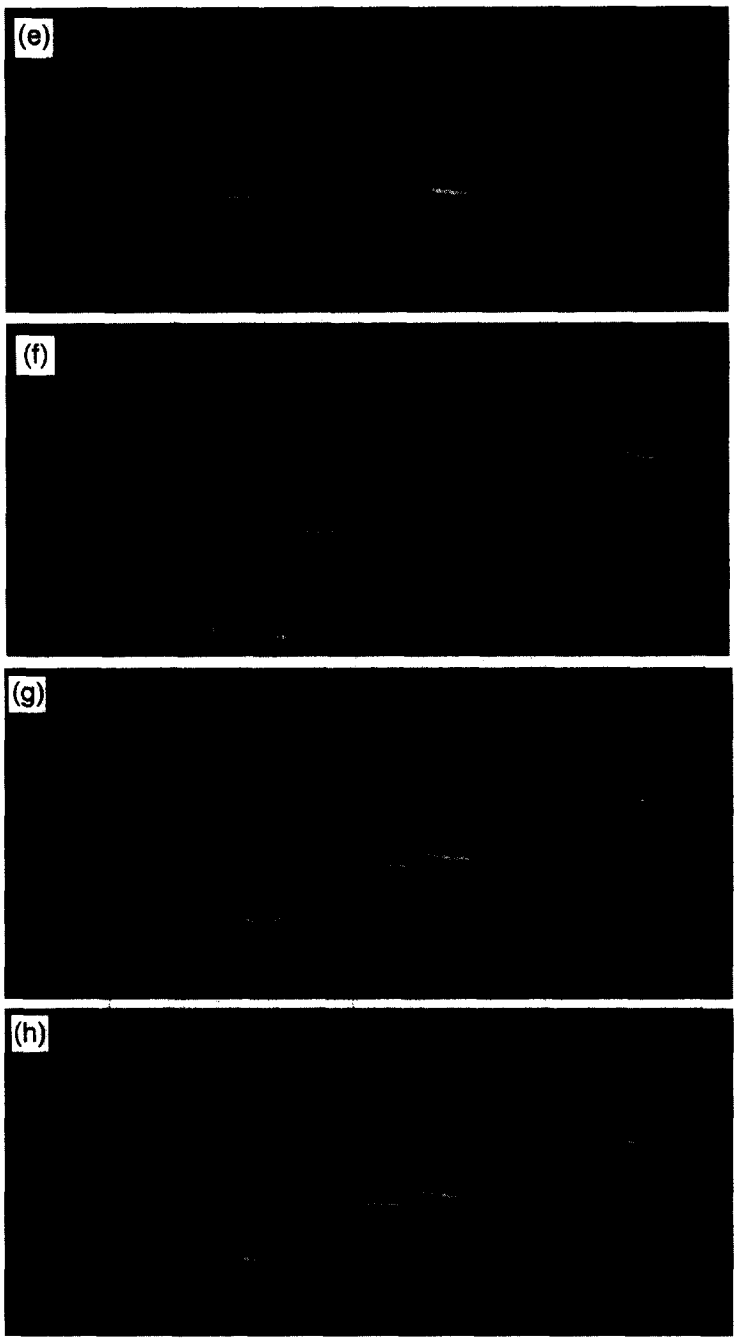

Fig. 1 (continued).

around the crystal favouring more self-regulated growth.

The vapour-diffusion method involves a phase boundary between the protein solution and the air space. This phase boundary is a source of another form of convection, i.e. Marangoni convection [7]. Marangoni convection in a hanging/sitting drop apparatus results from a difference in concentration or

temperature through the drop causing a difference in surface tension at the phase boundary.

This report describes the CCD observation of apocrustacyanin C_1 crystals during growth, and thereby documents the presence of Marangoni convection during crystallization in microgravity and shows depletion of the coloured protein (as protein is incorporated into the crystal) around the growing crystals.

2. Materials and methods

2.1. Apocrustacyanin

The blue colouration of the carapace of the lobster *Homarus gammorus* is produced by the astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) binding protein, α-crustacyanin. The native carotenoprotein is an aggregate of 16 apoprotein units each of about 20 kDa. The apoprotein consists of five electrophoretically distinct components of two main types C_1, C_2 and A_1 (type I) and A_2 and A_3 (type II) [8]. Apocrustacyanin C_1 was prepared as reported previously [9] and stored under 80% saturated ammonium sulphate for 4 days prior to use. Following dialysis against 0.1M tris-HCl, 1mM EDTA (pH 7.0) the apoprotein was concentrated using an Amicon Centricon-3 Anachem microconcentrator [2]. The apoprotein is faintly coloured blue due to some residual astaxanthin in the solution.

2.2. Crystallization

Crystallization of the protein [2] took place by the vapour-diffusion method in the European Space Agency (ESA) Advanced Protein Crystallization Facility (APCF) [10,11]. Each APCF facility carries 48 individual crystallization reactors consisting of three types; vapour-diffusion, liquid–liquid diffusion and dialysis reactors. The vapour-diffusion reactor consists of a reservoir formed by two porous, ultra high molecular weight, polyethylene blocks (each taking 0.35 ml of solution) and protein solution held in a glass cylindrical tube which can be raised to activate the crystallization process. The protein solution (20 mg ml^{-1} in 0.1M Tris-HCl, 1mM EDTA pH 7.0) was mixed 1/1 (v/v) with reservoir solution (5% (v/v) 2-methyl-2,4-pentanediol (MPD), 1mM EDTA, 0.1M Tris-HCl pH 9.0 and 1.9M ammonium sulphate) to form 50 μl drops. The APCF was flown on-board the International Microgravity Laboratory-2 (IML-2), STS-65, NASA space shuttle mission with a microgravity crystallization time of $12\frac{1}{2}$ days at a constant temperature of 20°C ± 0.1°C. Apocrustacyanin C_1 experiments totalled 8 reactors on the mission.

2.3. CCD observation

The APCF is fitted with a black and white, CCD video camera (582 lines, 500 pixels per line). This is constructed so as to observe, in sequence, 12 reactors out of a total of 48. Six can be monitored with a wide field of view system, six with a narrow field of view. The vapour-diffusion reactors (discussed here) are monitored with the wide field of view optics allowing an image 8.5 × 6.4 mm to be seen. The depth of focus of the CCD system is limited so a few images are taken at varying depths of focus through the drop formed in one reactor to survey the whole volume. Illumination is provided by an 850 nm LED and a polariser analyser system is available for birefringence tests. A sequence of 6 CCD images (at successive depth of focus) were recorded at intervals of every 7 h and 50 min starting 7 h and 21 min after initiation of the crystallization process until the final image at 12 days, 8 h and 51 min. Each image took between 4–5 min to record and write to the magnetic tape storage media. Two of the 8 apocrustacyanin C_1 reactors were monitored in this way using the CCD system. On completing the mission the APCF was retrieved from the shuttle.

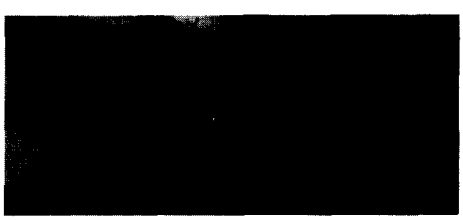

Fig. 2. Photograph taken immediately after landing illustrating the morphology of the crystals grown.

3. Results and discussion

Several single rod shaped crystals of apocrustacyanin C_1 were obtained with dimensions of 0.5–1.0 × 0.05–0.2 × 0.05–0.2 mm. Fig. 1 illustrates a time sequence of eight views (all at the same depth of focus) recorded from 46 h 32 min to 101 h 10 min. Three crystals are visible in each image. These are surrounded by a "halo" effect similar to what can be expected for a protein "depleted zone" around the crystals [3]. Several factors point to the observation of a depletion zone; the oval shape surrounding the needle like crystal (the needle shape resulting from the fact that growth is faster in the length

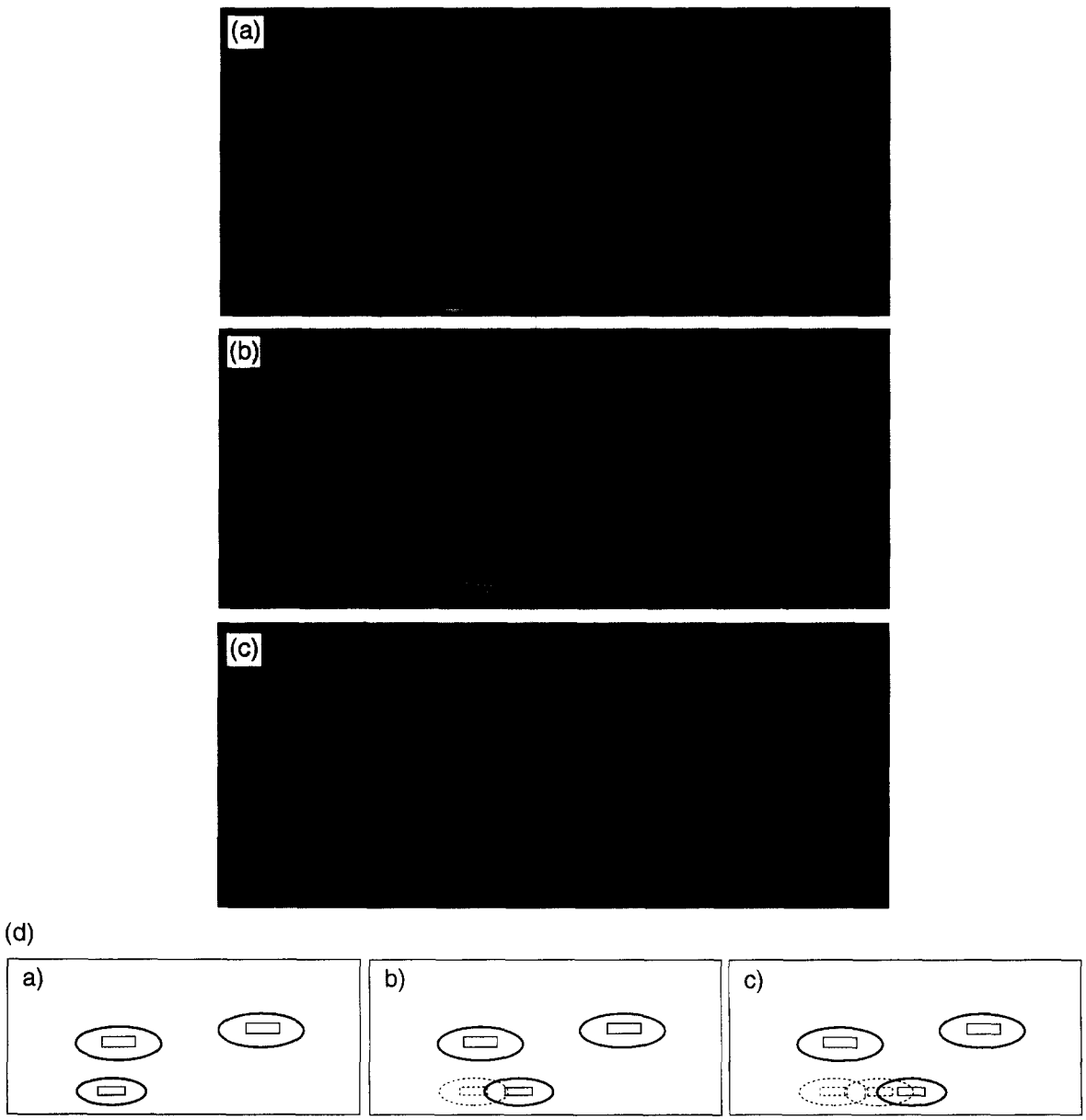

Fig. 3. (a, b, c) CCD video images taken at successive focal depths at times 85:30, 85:34 and 85:39 h. The arrows highlight one of the crystals which has changed its position. (d) Schematic diagram demonstrating the change of position of the crystal highlighted in (a)–(c).

direction than in the width hence the depleted region is more pronounced at the "tips" of the needle), the use of a coloured protein and duplication of the effect seen in two separate reactors. Note that the crystal habits are identical to final photographs taken at the end of the mission (Fig. 2). This corroborates the CCD monitoring method.

Also well illustrated with the CCD sequence of views is the movement of the crystals within the drops over time. In Fig. 1a–1g the crystal shown on the right hand side shows little motion. The other two crystals however are moving through the drop in an anti-clockwise rotation motion. This motion is rapid as illustrated in Fig. 3 with two images taken at subsequent focal positions approximately 5 and 10 min after Fig. 1f. Although the focus varies from each image it can be clearly seen that during the ~ 5 minutes between images the crystals are still in motion. It is not possible to uniquely label each of the crystals over time due to the low image sampling rate although a qualitative idea of the motion can be gained. The motion of the three crystals observed in the images is shown in Fig. 4, which is a plot of the crystal centre of gravity in each image in which a crystal is visible. One crystal is clearly separated from the rest, shown in the upper right hand side of the drop. Over time this moves in a shallow clockwise curve toward the right hand edge of the liquid/vapour boundary.

The motion of the crystals through the reactor is predicted by thermocapillary effects, i.e. Marangoni effects caused by temperature gradients. When the angle between the solid wall of the syringe tip and the liquid of the drop is less than 90° "roll cells"

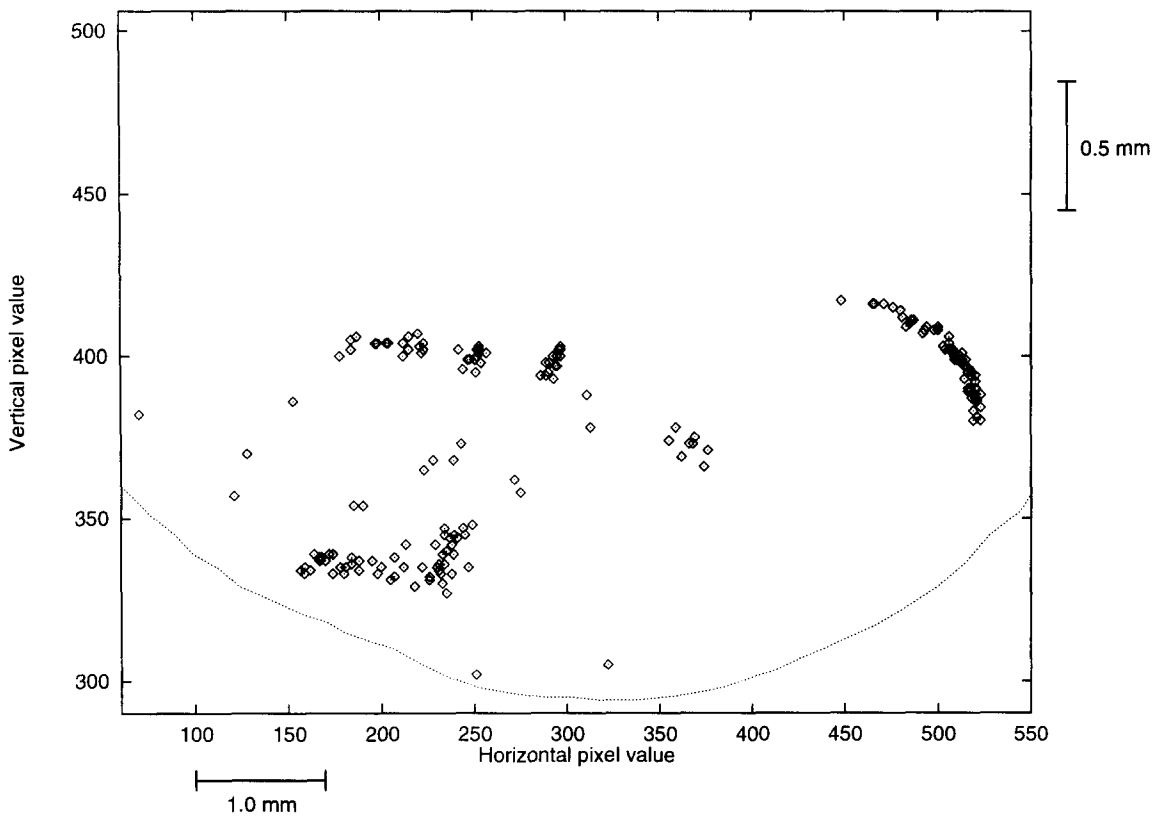

Fig. 4. Plots of crystal position (centre of gravity) within the drop throughout CCD images (frames (a)–(h) in Fig. 1) recorded during the mission. Coordinates are in pixels with 0,0 being at the bottom left of the images seen in Fig. 1. The pixel size of the CCD camera used is 0.013 × 0.015 mm. The dashed line represents the drop/vapour interface. The points on the right hand side represent one crystal moving in a clockwise direction over time. The other group of points are from two crystals. These have an anticlockwise motion but due to the sampling time it is not possible to say which crystal is in which successive image.

develop in the drop caused by water evaporating from the drop cooling the interface [7]. X-ray analysis of the resulting crystals showed that statistically the microgravity-grown crystals were superior when comparing the same conditions and using identical apparatus [2]. Microgravity growth has produced improved crystals but the Marangoni effects in vapour-diffusion methods may well be a limiting factor in the ultimate perfection that can be obtained. This is in contrast to lysozyme grown in microgravity by dialysis (without a phase boundary) where crystal motion was not visible (work in progress) and there was a very clear increase in crystal quality [1].

The presence of "halos" observed surrounding the crystals is interesting. The presence and the effect of depletion zones around growing crystals, especially in microgravity, has been discussed (eg. [5,6]) but only actually observed, as far as the authors are aware, in studies using gel crystallization [12]. The blue colour of the protein neatly shows up the absence of protein in the solution around the growing crystals by ordinary visible means. We believe that the presence of the "halos" provides, for the first time, evidence for the depletion zone around the growing crystals as is expected in a microgravity environment.

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