Acta Cryst. (1996). D52, 156–159

Crystallization of Apocrustacyanin C_1 on the International Microgravity Laboratory (IML-2) Mission

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(Received 25 March 1995; accepted 13 June 1995)

Abstract

Rod-shaped crystals of apocrustacyanin C1 have been grown under microgravity on the International Microgravity Laboratory (IML-2) NASA space shuttle mission using the vapour-diffusion set-up of the Advanced Protein Crystallization Facility (APCF). The crystals obtained under microgravity are compared with crystals grown simultaneously in ground control experiments in identical APCF reactors, and with those obtained in the laboratory. The degree of reproducibility of the results in microgravity was also tested. Statistically, the microgravity-grown crystals are larger and of better X-ray diffraction quality than those grown in the ground controls but inferior to the best crystals grown in sitting drops, in the laboratory. Diffracting crystals, the best to 2.3 Å, were produced in seven out of the eight reactors in microgravity, whereas the eight ground control reactors yielded only one poorly formed crystal suitable for diffraction studies, which also diffracted to 2.3 Å. The crystals belong to the space group $P2_12_12_1$ with two subunits per asymmetric unit.

1. Introduction

The blue cryptic colouration of the lobster carapace is provided by the astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) binding protein, α-crustacyanin. The absorption spectrum of the carotenoid in the complex is bathochromically shifted by some 150 nm, similar to the spectral shift for retinal in the visual pigment, rhodopsin (Wald, Nathanson, Jencks & Tarr, 1948).

The native carotenoprotein is an aggregate of 16 apoprotein units of about 20 kDa, one astaxanthin molecule being bound per apoprotein monomer. 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) (Quarmby, Norden, Zagalsky, Ceccaldi & Daumas, 1977); both types of apoprotein are members of the lipocalin family of small hydrophobic ligand binding proteins (North, 1991).

Extensive crystallization experiments have so far failed to yield well ordered crystals of the carotenoprotein α-crustacyanin. An improvement in molecular ordering has, for some proteins, been achieved by carrying out crystallization under microgravity (*e.g.* McPherson, 1993; DeLucas *et al.*, 1994). This has been attributed to lack of sedimentation and convection in microgravity. The elimination of convective mixing offers a slower, more homogeneous growth medium compared to growth on earth, and is therefore likely to improve the degree of perfection of the crystals. It is also conceivable that fewer nuclei will form compared to an environment stirred by convection. Moreover, in microgravity the growing crystals can remain suspended in a liquid droplet, which provides a uniform growth environment, contrary to earth trials where the crystal is situated at the top or the bottom of the solution (Nauman, Snyder, Bugg, DeLucas & Suddath, 1985). Larger crystals of the oligomeric α-crustacyanin than have so far been obtained terrestrially were grown under microgravity conditions but no visible X-ray diffraction was obtained from these crystals (Zagalsky, Wright & Parsons, 1995). Crystals of type I subunit, C_2 diffracting to 2.2 Å have been attained terrestrially (Wright *et al.*, 1992). More recently the major subunit, C_1 has also been crystallized.

The present report compares the crystallization of apocrustacyanin C_1 under microgravity and terrestrially using otherwise identical conditions and identical crystallization reactors. The crystallization conditions were based on those used by C. E. Wright & E. J. Gordon (personal communication) to obtain crystals in hanging-drop experiments in the laboratory.

2. Materials and methods

2.1. *Preparation of protein*

Crustacyanin was extracted and purified from lobster carapace and apocrustacyanin C_1 prepared as reported previously (Keen, Caceres, Eliopoulos, Zagalsky & Findlay, 1991). The subunit was stored under 80%

saturated ammonium sulfate for 4 d prior to use. Following dialysis against 0.1 *M* Tris–HCl, 1 m*M* EDTA (pH 7.0) the apoprotein was concentrated using an Amicon Centricon-3 microconcentrator and filtered through a 0.2 μm Anachem filter. The same protein preparation was used for both microgravity and ground experiments.

2.2. *Crystallization*

Crystallization took place in the vapour-diffusion reactors of the Advanced Protein Crystallization Facility (APCF) manufactured by Dornier GmbH (Snyder, Fuhrmann & Walter, 1991; Bosch, Lautenschlager, Pothast & Stapelmann, 1992). Preparatory ground-control experiments were performed to determine the optimal conditions for crystallization in the APCF reactors. Crystallization in the APCF reactors required a higher concentration (≥1.85 *M*) of precipitant than that in Linbro plates (1.4 *M*). The time of crystal growth in the APCF was similar to that in Linbro plates, with harvestable crystals formed within the time limit (10–12 d) allocated for the microgravity experiment. Eight reactors were used in microgravity while eight identical reactors acted as ground controls. The protein solution (50 μl) was held in a closed glass cylindrical tube which could be raised to expose the protein drop to the interior of the chamber, thereby activating the crystallization process, and subsequently lowered to shield the drop from the interior of the chamber, thereby deactivating the process. The reservoir was formed by two porous polyethylene blocks lining the chamber and taking 0.35 ml of reservoir solution each. The reservoir solutions contained 5%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD), 1 m*M* EDTA, 0.1 *M* Tris–HCl pH 9.0 with variable amounts (1.8–2.0 *M*) of ammonium sulfate. The protein solution (20 mg ml^{-1} in 0.1 *M* Tris–HCl, 1 m*M* EDTA pH 7.0) was mixed 1/1(*v*/*v*) with reservoir solution to form the 50 μl drops. In order to obtain an indication of the reproducibility of the results, the reactors containing 1.9 *M* ammonium sulfate (optimal conditions for ground crystallization) and 2 *M* ammonium sulfate were carried out in triplicate and in duplicate, respectively. Loading was carried out at Giessen, Germany, and the APCF transported by the European Space Agency (ESA), at 293±1 K, to Kennedy Space Center, USA, for the shuttle flight. The experiments were commenced when microgravity conditions ($10^{-4}g$) aboard the shuttle (Columbia) were attained; this was 10 d following loading of the reactors and 16 d following the preparation of the apoprotein. Ground controls containing identical solutions to those in microgravity were activated (*i.e.* the protein drops became exposed to the reservoir blocks) at the same time as those aboard the shuttle. On ground, the drops were orientated as sitting drops. Crystallization proceeded for 12.5 d at 293±1 K; deactivation of the corresponding (ground and flight) experiments was performed 24 h prior to re-entry of the shuttle. Following landing, the APCF reactors were transported at 293±1 K to Giessen and thence back to the UK where analyses were performed. Three of the ground-control reactors (1.8, 1.85 and 1.95 *M* ammonium sulfate, respectively) reactivated soon after deactivation due to movement of the syringe holding the drop, probably resulting from air pressure in the leak-tight reactors. (In microgravity the reactors were clamped on activation/deactivation.) As a consequence the drops in these chambers were exposed to the reservoir blocks for a prolonged period. A further ground control (1.9 *M* ammonium sulfate), giving poor quality crystals, reactivated in transport to Leeds prior to analysis.

2.3. *Harvesting of crystals and X-ray diffraction*

The largest crystal from each reactor was mounted in glass Lindemann tubes. Data were collected on a Xentronics detector (Siemens) mounted on a Rigaku rotating-anode generator (45 kV, 60 mA with 200 μm focus). Crystals were cooled at 278 K. Data were produced using *XDS* (Kabsch, 1988) and further analysis carried out with the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994) *ROTAVATE*, *AGROVATA* and *TRUNCATE*.

3. Results and discussion

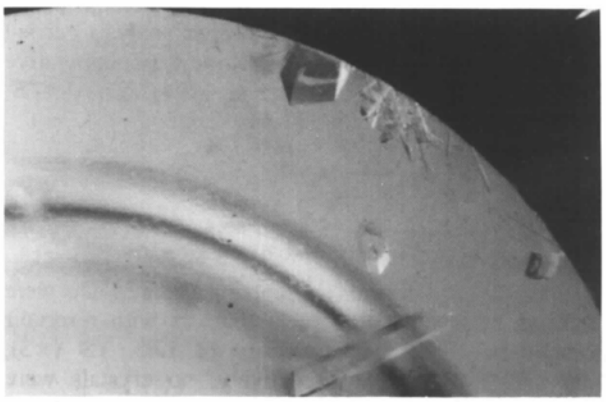
In microgravity crystals of apocrustacyanin C_1 were obtained in seven of the eight reactors with reservoir ammonium sulfate concentrations of 1.85, 1.9 (×3), 1.95 and 2.0 *M* (×2), respectively; no crystals were obtained in the reactor containing 1.8 *M* ammonium sulfate. The crystals were single and rod-shaped with dimensions of 0.5–1.0 × 0.05–0.2 × 0.05–0.2 mm, and diffracted to between 2.3 and 3.2 Å. The best crystals were formed, reproducibly, in the (triplicate) reactors containing 1.9 *M* ammonium sulfate. Microgravity conditions did not eliminate growth of crystals adhering to the wall of the glass piston holding the drop; these crystals were of inferior morphology to those in the bulk solution.

Crystals were likewise formed in seven of the eight ground controls (1.85–2.0 *M* ammonium sulfate) but were generally of poor morphology and size. In the four ground controls that did not function properly the crystals had a better chance to grow as they had more time in contact with the reservoir blocks compared to the other reactors, but in spite of this apparent advantage, the crystals were of poor quality. Only one crystal (0.75 × 0.25 × 0.1 mm), harvested from one of the three reactors containing 1.9 *M* ammonium sulfate, was suitable for X-ray diffraction analysis; data from this crystal was collected to a resolution of 2.3 Å, similar to that given by crystals grown in microgravity using the

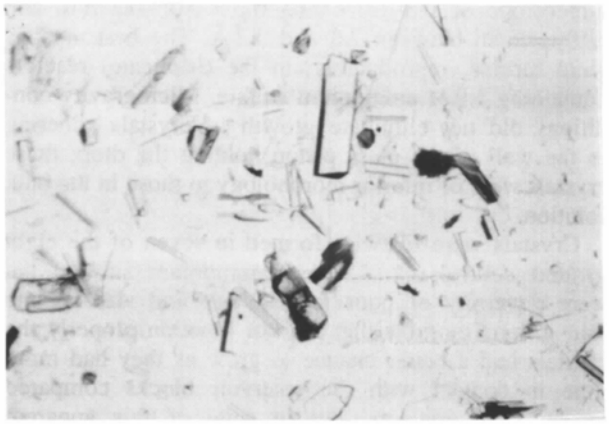
same ammonium sulfate concentration in the reservoir. The crystals exhibit the same symmetry ($P2_12_12_1$; cell dimensions: $a = 42.0$, $b = 81.9$, $c = 110.9$ Å) as crystals grown in the laboratory in hanging and sitting drops (Gordon & Wright, personal communication). A comparison of typical crystals grown in microgravity and in the ground controls is shown in Fig. 1.

It is still not established whether the optimum terrestrial conditions for crystallization of proteins are also optimal for crystallization under microgravity. Experiments conducted by Strong, Stoddart, Arrott & Farber (1992) using batch method for the crystallization of hen egg-white lysozyme, suggested that the optimal protein concentration for growth in microgravity is higher than that on earth. Another study implied that the enzyme reverse transcriptase required a higher concentration of precipitant for crystallization in microgravity (Chayen, 1995). In the present crystallizations at fixed protein concentration, there appears to be no shift in the optimal concentration of precipitant between ground and microgravity experiments, with the best crystals grown from 1.9 *M* ammonium sulfate in both environments. Moreover, the same form of crystal is produced in microgravity, ground controls and in the laboratory, contrary to a number of cases where a different crystal form was obtained in microgravity (*e.g.* Asano, Fujita, Senda & Mitsui, 1992; Stoddart, Strong, Arrott & Farber, 1992).

Even though a single ground-control crystal diffracted to as high a resolution as those grown under microgravity, statistically the microgravity-grown crystals were superior when comparing the same conditions and using identical apparatus. However, the microgravity-grown crystals are inferior to the best crystals grown in the laboratory in sitting drops. Prior to the IML-2 mission, the best crystals grown in the laboratory (in hanging drops) diffracted to 2.3 Å. Since the flight, and after numerous trials to perfect conditions in the laboratory, crystals diffracting to 1.6 Å have recently been obtained in sitting drops (Gordon, personal communication).



(*a*)



(*b*)

Fig. 1. Comparison of microgravity- and earth-grown crystals. (*a*) Microgravity-grown crystals. Reservoir: 2 *M* ammonium sulfate, 5% MPD, 1 m*M* EDTA and 0.1 *M* Tris–HCl (pH 9). The appearance of the crystals shown is typical of all microgravity reactors (1.85–2.0 *M* ammonium sulfate). (*b*) Earth-grown crystals (ground control). Reservoir: as in (*a*). The appearance of crystals is typical of the ground-control reactors. Scale 1.1 cm = 0.5 mm.

The authors thank the European Space Agency (ESA), especially Dr K. Fuhrmann and Dr H. U. Walter, for the opportunity to crystallize samples under microgravity, and we thank Dr R. Bosch of Dornier for valuable practical assistance. NEC and PFZ acknowledge with gratitude financial support for travel received from the BBSRC. EJG acknowledges support awarded to Professors A. C. T. North, J. B. C. Findlay and S. E. V. Phillips by the SERC (now EPSRC).

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