Crystallization of a human Bence–Jones protein in microgravity using vapor diffusion in capillaries

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Abstract

A simple evaporative method in sealed capillaries was used to produce X-ray diffraction quality crystals of a monoclonal human Bence–Jones protein (Sea) in microgravity and at unit gravity. The \( \lambda \) isotypic Bence–Jones protein was purified from the urine of a multiple myeloma patient using ammonium sulfate precipitation, dialysis and cation exchange, followed by gel filtration. Crystals were produced in glass capillaries where water evaporated from the protein solution was absorbed by one or two suitable absorbents. A crystal grown during the 9-day Space Shuttle STS-95 flight measured 8 \( \times \) 1.6 \( \times \) 1 mm. It was subjected to X-ray diffraction and was found to be orthorhombic (\( P2_12_12_1 \)), with unit-cell dimensions of 48.9, 85.2 and 114.0 \( \AA \). X-ray data were collected at room temperature and were 98.3\% complete to 2.3\( \AA \) resolution. Crystals of the same Bence–Jones protein measuring 1.2–2 mm in length were grown in ground-based controls using a high evaporation rate for the first 12 h, followed by a slower evaporation rate for the remainder of the 19-day growth period. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In multiple myeloma monoclonal antibodies consisting of two light (L) and two heavy (H) chains are produced by malignant plasma cells and then secreted into the bloodstream. L chains (\( \kappa \) or \( \lambda \) isotypes) with amino acid sequences identical to those in the intact antibodies are overexpressed in relation to the H chains in about one-third of the myeloma patients. After secretion into the bloodstream as monomers or disulfide-linked homodimers, these L chains are usually cleared or destroyed by the kidney within 5 h. When excreted into the urine, the L chains are called Bence–Jones proteins after the British physician, Henry Bence–Jones, who first described them as pathognomonic of “myelomatosis” in 1848 [1].

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In about 10% of the cases, the L chains pass through the endoplasmic lining of the blood vessels into the periplasmic space and precipitate as pathological amyloid deposits. Participation in amyloid fibril formation favors λ over κ chains by a 3:2 margin in human subjects [2]. Amyloidosis of L chain origin (AL) is morphologically similar to the fibrillar amyloid deposits found in 18 or more diseases, like diabetes and Alzheimer’s disease. Irrespective of their sources, amyloid deposits appear to contain unbranched fibrils 80–110 Å across and of variable length, with β-pleated sheets as their principal underlying structures [3].

The covalent Bence–Jones dimer, produced in vivo by a patient (Sea) with multiple myeloma and amyloidosis, was previously shown by an in vitro assay to form fibrillar amyloid deposits in fibroblast monolayer cell cultures [4]. Fine filaments interspersed with nascent collagen could be detected after 48 h. In 72 h, the deposition of L chains culminated in the appearance of dense amyloid fibrils. Formation of these fibrils was accompanied by interference with the maturation of the collagen synthesized by the fibroblasts. Fibrils of the Sea Bence–Jones protein deposited between collagen fibers, expanded them laterally and led to their partial disintegration. This process prevented the collagen from maturing. In a control experiment, collagen developed normally in cultures exposed to a non-amyloidogenic Bence–Jones protein (Hud). The amyloidogenic Sea Bence–Jones protein was demonstrated to decorate nascent collagen strands by immunochemical techniques with fluorescein- and gold-labeled anti-L chain antibodies.

Duplication of these results with three other amyloidogenic Bence–Jones proteins (Mcg, Bla and Jen) supported the conclusion that these pathological L chains are concentrated in the extracellular matrix by monovalent antigen–antibody type reactions. Participation in such interactions is self-destructive. Amyloidogenic L chains subsequently lose their binding capabilities for collagen-derived peptides and by a still undefined mechanism are irreversibly converted to amyloid fibrils. We found that all of these dire events could be prevented by prior treatment of the amyloidogenic L chains with ligands that effectively blocked their active sites [4]. In crystals of the Mcg dimer, the most effective of these active site blockers was a collagenase substrate [5], PZ-Pro-Leu-Gly-Pro-D-Arg, where PZ is a 4-phenylazobenzylhydroxycarbonyl moiety.

In the present article we describe the crystallization of an amyloidogenic Bence–Jones protein (Sea) by vapor diffusion in capillaries. Under microgravity conditions, crystals formed in profusion within a single capillary. Sequestered within the shower of crystals was a very large single crystal of the Bence–Jones protein. In ground-based experiments, the technique was modified to allow the operator to control the rate of nucleation and crystal growth. The laboratory experiments repeatedly produced large single crystals of the Bence–Jones protein.

2. Experimental procedure

2.1. Preparation and purification of the Sea Bence–Jones protein

Urine samples were obtained from a patient (Sea) with multiple myeloma and amyloidosis. The Bence–Jones protein was precipitated from the urine by adding solid ammonium sulfate to 75% saturation and the precipitate was recovered by centrifugation at 5000 RPM for 20 min. This precipitate was washed three times with the 75% ammonium sulfate solution and collected each time by centrifugation. The ammonium sulfate–protein paste was stored at −10°C until needed. Purification for subsequent crystallization trials began by dissolving paste samples containing 50–100 mg of protein in 15 mM TRIS, pH 9.2, containing 0.05% sodium azide, and dialyzing against the same buffer overnight at 4°C.

This protein solution was loaded onto a column prepared with 20 g of Whatman DE52 (DEAE-cellulose) that had been equilibrated thoroughly with the starting buffer (15 mM TRIS, pH 9.2). If the column was overloaded with protein, a large component emerged without significant retardation. By polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS), this large fraction was found to be composed...
predominantly of dimers of the Bence–Jones protein held together by interchain disulfide bonds ("covalent dimer"), plus a small quantity of aggregated protein (<5% of the total). Even with an overloaded column, a considerable proportion of the starting material was retained on the DEAE-cellulose. The column was washed with 250 ml of the pH 9.2 TRIS. To elute the bound protein, a linear gradient was established with pH 9.2 TRIS placed in one vessel and 40 mM TRIS, pH 8.0 in the other. A major band with a yellow-green tint and a smaller peak appeared on the chromatogram. Again, the major component consisted primarily of the Sea covalent dimer, while the smaller fraction contained at least two other contaminants that absorbed ultraviolet light at 280 nm. These conclusions have all been confirmed by SDS-PAGE on 10% gels.

Fractions containing the Sea dimers were further purified by gel filtration at 4°C on a 2.5 × 95 cm column of Pharmacia Sephacryl-200, equilibrated with 0.1 M TRIS-0.5 M NaCl, pH 7.7. An initial aggregate band representing only 1–2% of the protein was followed by a very large symmetrical peak, with a yellow component appearing as a shoulder on its descending slope. Fractions were pooled in such a way as to avoid the colored contaminant, which is an unidentified urinary pigment non-covalently adhering to the Sea dimer. While not entirely preventing crystallization of the Sea protein, the pigment retards the appearance of crystals with sharply defined edges.

2.2. Methods used for the crystallization of the Sea Bence–Jones protein

The Sea Bence–Jones protein is very soluble in aqueous media over a wide range of pH values. Before crystallization, the eluate from the Sephacryl column could therefore be dialyzed against many buffers or weak salt solutions of choice. A Spectrum vacuum concentrator was used for this dialysis and the simultaneous concentration of the protein sample. In our most successful experiments, the purified Bence–Jones protein was dialyzed against 0.05 M NaCl-0.05% sodium azide and concentrated to 40–45 mg/ml. Four basic procedures have been employed, all with polyethylene glycol (PEG) as the precipitating agent: (1) vapor diffusion in hanging or sitting drops, with a starting concentration of 5–6% (w/v) PEG 8000 in the droplet on average and a reservoir solution of 10–12% PEG 8000; (2) a microseeding procedure [6], in which microseeds prepared from spontaneously appearing protein crystals are added to solutions of the Sea Bence–Jones protein (45 mg/ml) in 7–10% PEG 8000; (3) osmotic dewatering [7], which was used under microgravity conditions in the US Shuttle flight STS-80, with 4% PEG 8000 inside the capillary and 8% PEG 8000 in the reservoir that was separated from the capillary by an osmotic membrane; and (4) vapor diffusion in capillaries, employed for the production of crystals of the Sea Bence–Jones protein under microgravity conditions in the US Shuttle mission STS-95 and in current ground-based experiments. The apparatus for methods 3 and 4 were made available by Instrumentation Technology Associates, Inc., Exton, PA. Application of procedure 4 is the major topic of the present report.

2.3. Vapor diffusion in capillaries

The method used to grow the Bence–Jones protein crystals was based upon vapor diffusion in capillaries. The use of capillaries offers several advantages, including: (1) the compactness of the equipment, which is very important for microgravity-based experiments; (2) the mechanical stability of the system, which is critical for high-vibration situations during launch and reentry; (3) the ability to use material that is optically transparent, thus enabling observation of the growing crystals and the convective/diffusive environment at the liquid–solid interface; and (4) the ability to grow the crystals in quartz capillaries, suitable for subsequent examination of the crystals using X-ray diffraction. It was recently demonstrated by Lopez-Jaramillo et al. [8] that quartz capillaries can be adapted for cryo-crystallography, thereby nearly eliminating the possible damage of the crystals by mechanical transfer to a loop for freezing. The adaptability of the capillary method and its application to the "gel acupuncture" technique have been demon-
strated by Garcia-Ruiz, Moreno and colleagues [9].

In the present experiments, sealed capillary tubes, 6 cm in length and 1.6 mm in internal diameter were arranged in the basic configuration shown in Fig. 1. Note that the internal diameter of the capillaries is too great (> 1 mm) to prevent convection in ground-based experiments [9]. However, in microgravity convection is practically eliminated and diffusion is the predominant mechanism for the transport of components through the crystallizing solutions. Variable quantities of the protein solution were placed in the capillaries, mixed with solutions of crystallizing media, prepared at lower concentrations than those required to crystallize or precipitate the protein. This column of protein solution was separated from a liquid absorbent by an air gap that was set at a constant length for each set of experiments. Lengths of air gaps were standardized because it has been shown that the transport of water vapor across an air gap, in sealed capillaries, is an important rate-determining step in the procedure [10].

In the course of the experiments, water from the protein solution is transferred across the air gap by vapor diffusion and trapped by the absorbent. The evaporation and absorption processes increase the concentrations of the protein solution and its appropriate crystallizing agent. Concomitantly, the absorbent is diluted. During the evaporation process the air column remains constant in length and migrates from the absorbent column toward the protein solution. For the solutions used in the present studies, the 1.6 mm internal diameter of the capillaries ensured sufficient surface tension to prevent liquid-to-liquid contact between the protein and absorbent columns, even under the harsh vibration and acceleration conditions of ascent and reentry in space vehicles.

A single absorbent was used for each capillary tube set up for microgravity-based experiments. Absorbents included 100, 80 and 66% glycerol and 20% NaCl. The strongest absorbent was 100% glycerol, which established the fastest evaporation rates and was used in the only successful space experiment. In ground-based experiments, a step function in evaporation rate was obtained by sequentially exposing the protein solutions to a strong absorbent (typically 100% glycerol), followed after a timed delay to a weak absorbent (e.g., 80% glycerol). The advantage of this procedure was that the nucleation and crystal growth steps could be largely controlled, allowing only a few single crystals to appear in each capillary tube.

3. Results

3.1. Crystals of the Bence-Jones protein produced using various procedures

Crystals from all four procedures fell into two types, groups of long, platy crystals emanating in a "starburst" pattern from a common stalk and individual thicker rods (see Fig. 2). Whereas the crystals cut out of the starbursts were of ample length and width (1–4 × 0.3–0.5 mm), they were only 0.05–0.12 mm thick and therefore were best suited for analysis using synchrotron radiation. Such crystals were the predominant forms obtained by osmotic dewatering (procedure 3) in the STS-80 space shuttle mission (Fig. 2). In this method, glass capillaries were protected in a polycarbonate plastic lined stainless steel holder
within a Liquids Mixing Apparatus (LMA). Individual crystals were more common in hanging or sitting drop vapor-diffusion (procedure 1) experiments, microseeding procedures (2) and the vapor diffusion in capillaries (procedure 4), used in the STS-95 space shuttle mission.

3.2. Bence-Jones protein crystals obtained during US Space Shuttle mission STS-95

Fig. 2 shows a photograph of an STS-95 protein crystal 8 mm long, 1.6 mm wide (this dimension was limited by the internal diameter of the capillary) and 1 mm thick, one of the largest crystals known to be produced in space using evaporative techniques. This crystal of the Sea Bence-Jones protein appeared in one of the 64 samples of Bence-Jones proteins and other antibody fragments placed on board STS-95 by ABE. It is remarkable that a crystal this large could be grown during the relatively short flight period of only 9 days. In ground-based experiments, it usually requires 19 days for the appearance of crystals 10 times smaller. The capillary configuration was modeled on one shown in Fig. 1. We used 54 µl of a solution of 45 mg/ml of the Sea Bence-Jones protein in 4% PEG 8000, an air gap of 10 mm and 20 µl of 100% glycerol.

In the capillary, the protein section was literally jammed with crystals, some of which even protruded into the air gap. More than 300 smaller crystals surrounded the large one. Many of these were suitable for data collection at synchrotron facilities. Long thin rods (>1.0 x 0.2 x 0.1 mm) abounded and we are currently developing procedures for prolonged storage of crystals appearing in such unexpected deluges until adequate synchrotron time can be arranged.

Despite extensive variations in the protocols to optimize probabilities for obtaining crystals, only one of 20 capillaries containing the Sea Bence-Jones protein yielded crystals during the STS-95 mission. Forty-four capillaries containing other antibody fragments also were devoid of crystals at the time of recovery. All 63 of the unsuccessful experiments were designed with conditions that had routinely produced crystals (~10 to >100 crystals per capillary) in ground-based experiments. The successful sample contained the largest quantity of protein and the most powerful dehydrating agent (100% glycerol) in the series. Both decisions were very extreme by comparison with the rest. These extreme conditions may have been necessary considering the length of the space flight, which in STS-95 was only half as long as the shortest productive crystallization trial, using more conservative conditions, conducted for the Sea protein in our home laboratory (see below).

In the evaluation of the crystals from STS-95, we again took a conservative approach. The large crystal of the Sea Bence-Jones protein was cut into
pieces, one of which measured 1.0 × 0.5 × 0.3 mm. When this fragment was subjected to X-ray analysis at ambient temperature (20°C), the Sea protein was found to crystallize in the orthorhombic space group P2₁2₁2₁, with a = 48.9, b = 85.2 and c = 114.0 Å; α = β = γ = 90°; the crystallographic asymmetric unit consisted of one dimer. X-ray data were collected on a Rigaku rotating anode/R-axis IV image plate detector system operated at 50 kV and 90 mA. Initially, reflections extended to the edge of the detector (d spacings of 1.9 Å). However, the crystal was very sensitive to X-ray damage and the data were truncated to give a set ranging from 25 to 2.3 Å. The degree of completeness was 98.3% and the Rmerge was 7.7%. Values for I/σ(I) were 16.1 for all reflections and 7.6 for those in the highest resolution shell (2.38–2.30 Å). Irrespective of the methods chosen, crystals of the Sea Bence-Jones protein produced in the home laboratory and in space all retained the same space group and unit cell dimensions. The three-dimensional structure of the Sea Bence-Jones protein, crystallized in space, was solved at 2.3 Å resolution by the molecular replacement method [11,12].

3.3. Ground-based experiments

Experiments using a modified procedure were performed at the Oklahoma Medical Research Foundation. Starting solutions contained 43 mg/ml of the Sea Bence-Jones protein (20–50 μl volumes), 4–6% PEG 8000 and 0.05 M sodium chloride. A different configuration was employed for the capillary system, in which two adsorbent concentrations were sequentially exposed to the protein solution. During the initial 12 h the strongest adsorbent, 100% glycerol, was applied across a 10 mm air gap. After this initial rapid evaporation phase, the 100% glycerol was removed and was replaced at the other end of the capillary tube by 80% glycerol, which was maintained for the remainder of the 19 days of the experiment. After 19 days of evaporation in the home laboratory, we obtained the single crystals shown in Fig. 3. These crystals measured 2.0 × 0.5 × 0.25 mm (larger crystal) and 1.2 × 0.45 × 0.15 mm (smaller crystal). Crystals were obtained in 18 of 20 capillary tubes that were sequentially exposed to the two adsorbents. A single crystal of the Sea Bence-Jones protein,

Fig. 3. Crystals of the Sea Bence-Jones protein produced in a capillary in a ground-based experiment. The protein solution was first exposed to 100% glycerol for 12 h, after which it was replaced by 80% glycerol for the remainder of the 19 day growth period.
grown using the above technique, was used to collect an X-ray data set for comparison with the crystals produced in microgravity. These analyses and structures will be reported separately (S. Terzyan, P.C. Bourne and A.B. Edmundson, manuscript in preparation).

4. Discussion

We report here the crystallization, in microgravity and at unit gravity, of a human Bence–Jones protein using the method of vapor diffusion in sealed glass capillary tubes. Capillary tubes have been used for crystallization of macromolecules by methods based upon liquid–liquid diffusion, liquid–gel diffusion and vapor diffusion (reviewed in Refs. [9,13]. Zepezaue et al. (1968) pioneered the use of capillaries for growing single protein crystals in thick-walled capillaries that were capped at one end and covered by cellophane dialysis membranes at the other [14].

Double-diffusion methods in capillaries have also been successfully implemented in a microgravity environment on the Russian space station Mir. For these experiments tubes were filled, half with the protein solution and the other half with the crystallizing media, and flash-frozen in liquid nitrogen. The capillaries were kept frozen (called "a popsicle arrangement" by A. McPherson) until transported into space, where they were allowed to thaw and crystallization occurred by liquid–liquid diffusion [15]. García–Ruiz’s group [9] has developed methods for the growth of protein crystals, suspended in gels, in small bore glass capillaries. In their hands, the most successful experimental design involves using vertical capillaries sealed at one end and where the open end is punctured into gel/precipitant reservoirs (i.e., the "gel acupuncture" technique). The rate of diffusion can be controlled by the internal diameters and lengths of the capillaries, the type and density of the gel matrix, as well as more conventional variables such as the concentrations of the protein and precipitating agent.

An important rate determining element of vapor diffusion methods is the transport of the vapor across the air gap [10]. Therefore, in order to avoid problems associated with different vapor transport rates between the protein solution and the absorbent, we set a constant size for the air gaps in our glass capillaries (normally 10 mm lengths) for each set of experiments. Instead of varying the volume of the air gap we adjusted the relative strengths of the adsorbents, to control the rates and extent of evaporation of the protein solutions.

In the 9 day space flight (STS-95) experiment the only successful experiment used the largest quantity of Bence-Jones protein and the strongest adsorbent (100% glycerol). In ground-based experiments, a strong adsorbent (100% glycerol) was used to promote nucleation and a weaker adsorbent (80% glycerol) was used to maintain crystal growth. These results support previous comparisons of microgravity and unit gravity, where it is generally agreed that crystallization often requires higher concentrations of protein and/or precipitant for short-duration microgravity experiments than for ground controls (for review see Ref. [13]). However, even in such a short flight we produced a very large crystal of the Bence–Jones protein, with a length of 8 mm. The more conservative protocols followed in the laboratory required twice as much time to produce crystals with lengths of 1–2 mm.

5. Conclusion

We have applied vapor diffusion in capillaries to produce single crystals of a medically important human protein. Crystals of an amyloidogenic Bence-Jones urinary protein (Sea) were produced both in space and on the ground. The crystals grown in space diffracted X-rays to d spacings of at least 1.9 Å and a near complete diffraction set to 2.3 Å resolution was collected at room temperature. Similar crystals were grown, in ground-based experiments, using a modified protocol where two adsorbents were used to control the nucleation and crystal growth steps. These crystals have the same orthorhombic P2_12_12 space group with unit-cell parameters very similar to those of crystals prepared under microgravity conditions. The X-ray structures of the Sea Bence–Jones protein are in the final stages of crystallographic refinement and these studies will be reported separately.