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Freezing of Myoglobin Crystals at High Pressure

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A method is described for freezing sperm-whale myoglobin single crystals at a hydrostatic pressure of 2500 atm. Precession photographs show no damage to the lattice order of the frozen crystals.

1. Introduction

Crystallographic phase determinations in protein single crystals by means of nuclear γ-resonance scattering meet with considerable difficulties (Parak, Mössbauer & Hoppe, 1970). One of the problems is the low recoilless fraction (f factor) of the nuclear-scattering process at room temperature. For sperm-whale myoglobin it has been shown that at room temperature the probability of γ-resonance scattering at a ^{57}Fe nucleus is only 1 % of the theoretical value, which is equivalent to a scattering amplitude of 490 electrons (Parak & Formanek, 1971). To increase the recoil-free fraction one must cool the crystal. Unfortunately, freezing protein single crystals is a major problem since the crystal water expands during the liquid/ice phase transition, which usually destroys the single crystal.

Haas & Rossmann (1970) have published a method of freezing lactate dehydrogenase crystals by adding certain amounts of sugar to the mother liquid. In this way, they obtain during the freezing process some kind of glass, which does not rupture the crystal structure. The disadvantage of this method is the necessity to find for each protein the exact working conditions. Their aim was to reduce the radiation damage to the crystals during exposure to X-rays. They found that the rate of the radiation damage to the frozen crystals was ten times less than at room temperature. This effect should be expected for any freezing method.

In the following we describe a method of freezing sperm-whale myoglobin crystals. In order to prevent the damaging high volume change during the freezing process, one may work in a more suitable range of the water phase diagram. The so-called ice III and ice IX phases, which differ only in the ordering of the protons, exist at a pressure between 2100 and 3500 atm. The phase transition of water to the ice III phase is accompanied by a volume contraction in contrast to the expansion which occurs during the formation of the ice I at atmospheric pressure. Besides, the absolute alteration of the volume is considerably smaller (Whalley, Davidson & Heath, 1966; Whalley, Heath & Davidson, 1968; Riehl, Bullemer & Engelhard, 1969).

For these reasons, we have frozen sperm-whale myoglobin crystals at a hydrostatic pressure of 2500 atm and investigated the crystals at liquid-nitrogen temperature on a precession camera. The photographs can be taken at atmospheric pressure, because the high pressure phase is metastable at very low temperatures.

2. Experimental details

The high-pressure freezing equipment is shown in Fig. 1. Part 6 of Fig. 1 is removed at the beginning of the

freezing procedure. The pistons 2 and 3 are coupled to a conventional hydraulic press. The crystal floats in a liquid in a Teflon cell (detail 4 in Fig. 1). After slowly raising the pressure to 2500 atm the vessel No. 5 is filled with liquid nitrogen. After cooling, the pressure is relaxed and part 6 is placed into position shown in Fig. 1. Now one couples parts 3 and 6 to the press and pushes cell 4 out of the cylinder 1.

The liquid must transfer the high pressure hydrostatically to the protein crystal to prevent a rupture. Experiments at room temperature have shown that the X-ray picture of a myoglobin crystal after being exposed to 2500 atm was unchanged. Because it was not possible to prepare the crystal from an ice block of mother liquid, it was impossible to employ the mother liquid for the hydrostatic pressure transfer. Instead isopentane was used for this purpose. The surfaces of the crystal were carefully cleaned of other liquids before it was placed in the isopentane. Since this liquid is not soluble in water, it does not diffuse into the protein crystal. Crystals immersed for two months in

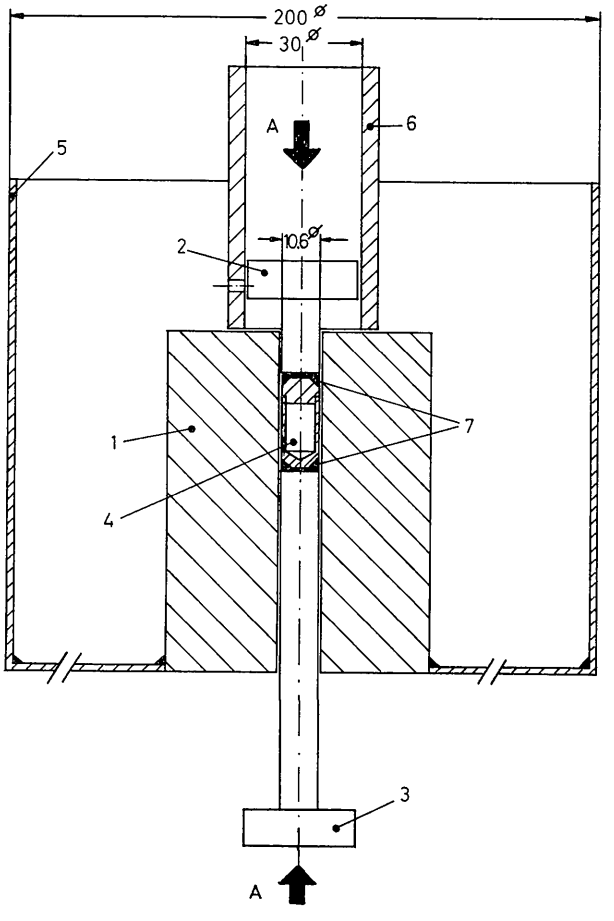

Fig. 1. Equipment for freezing at 2500 atm. (1) Steel cylinder, (2) and (3) pistons which can be coupled to a hydraulic press, (4) Teflon cell, (5) vessel for liquid nitrogen, (6) steel tube used for pushing cell (4) out of cylinder (1), (7) steel rings. Scale is in mm. *A–A*: direction of the force.

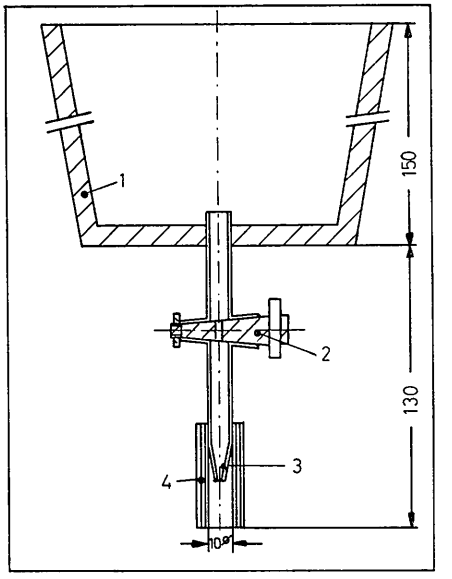

Fig. 2. Cooling equipment for the precession camera. (1) vessel of Styropor for liquid nitrogen, (2) throttle valve (Teflon and glass), (3) glass jet, (4) cellulose to prevent freezing of the glass jet. Scale is in mm.

isopentane did not show any change in the X-ray photographs. Isopentane forms at 77 °K a rather brittle solid and, therefore, it is not very difficult to clean this substance from the crystal.

In order to perform X-ray measurements, the crystal was fixed in a capillary with the aid of cellulose. The capillary was then mounted on a goniometer head. It proved rather difficult to transfer the crystal from the Teflon cell onto the precession camera, since the sample had to be kept constantly at liquid-nitrogen temperature to prevent a phase transition.

The crystals were investigated on a Nonius (Delft, Holland) camera. The simple device shown in Fig. 2 was used for the work at liquid-nitrogen temperature. First, the container 1 was filled with liquid nitrogen. A thin stream of liquid nitrogen controlled by the throttle valve 2 drops directly on the capillary. The constant stream of nitrogen onto the capillary prevents the deposition of ice in the region of the crystal. The capillary was covered with ice some millimeters from the X-ray beam, but this did not interfere with the operation of the equipment. The liquid nitrogen was recollected below the capillary.

3. Results and discussion

Fig. 3 shows a precession photograph at 77 °K of the (*hk*0) plane of a myoglobin crystal frozen at 2500 atm. Obviously, the crystal is not broken into smaller pieces. Reflexions are visible also at higher orders up to a resolution of 2·2 Å. This means that the microscopic disorder in the specimen is not appreciably increased by our freezing method. The precession photograph, essentially, does not differ from a room temperature

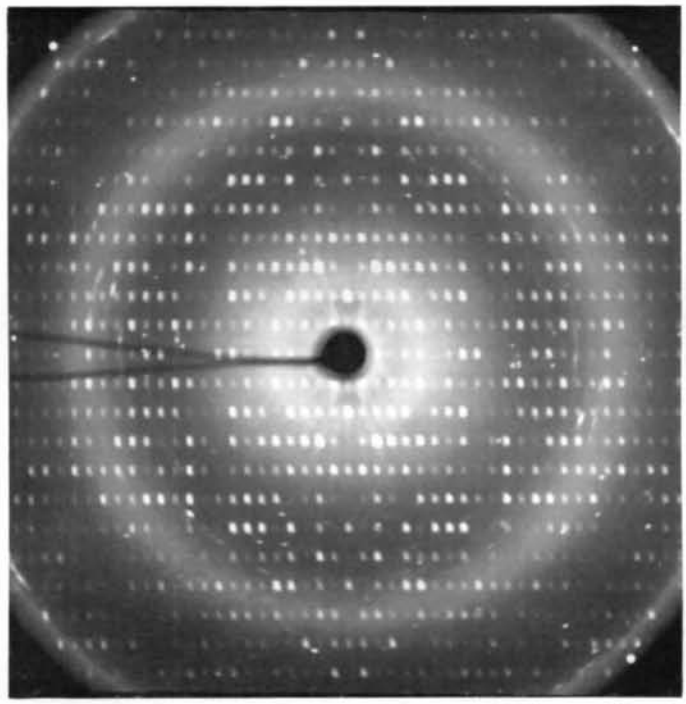

Fig. 3. Precession photograph of a sperm-whale myoglobin crystal frozen at 2500 atm. The photograph was taken at 77°K, $\mu = 21°$, ($hk0$) plane, film distance 100 mm, Cu $K\alpha$ radiation, exposure time 4 hours. The bright ring is due to the liquid nitrogen flowing over the capillary.

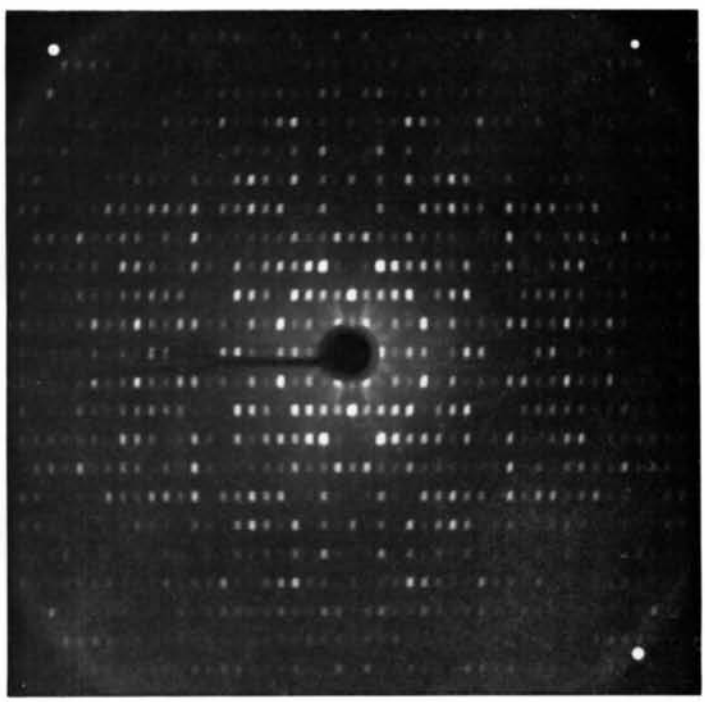

Fig. 4. Precession photograph of a sperm-whale myoglobin crystal taken at room temperature. The exposure time is 4 hours, $\mu = 21°$, ($hk0$) plane, film distance 100 mm, Cu $K\alpha$ radiation.

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photograph, which is shown in Fig. 4. Both photographs (Figs. 3 and 4) were taken with the same specimen.

The reciprocal-lattice vectors change during the freezing process. From Fig. 4 (room temperature, 1 atm) one obtains $a^*=0{\cdot}01606$ (3) and $b^*=0{\cdot}03215$ (6) $Å^{-1}$ and from Fig. 3 (77°K, frozen at 2500 atm) one obtains $a^*=0{\cdot}01619$ (3) and $b^*=0{\cdot}03214$ (6) $Å^{-1}$. The change of the reciprocal-lattice vectors is +8%.

The bright diffuse ring in Fig. 3 stems from the liquid nitrogen flowing over the capillary.

The phase transition from the metastable high-pressure phase to the normal phase, which occurs at roughly 120°K and at atmospheric pressure, is accompanied by a total destruction of the Mb crystal.

We now discuss some details of the freezing process. First of all one must consider the fact that the myoglobin crystal does not contain pure water but a 3·75 *M* $(NH_4)_2SO_4$ solution. For this reason the water phase diagram has to be modified. For pure water $\Delta_v/v_{\text{liquid}}$ equals −9% for the phase transition at 1 atm and +3·7% for the transition at 2500 atm.

Experiments showed that the volume alteration during the liquid/solid phase transition is smaller in the mother liquid than in pure water. To get a rough numerical value, we measured the heat of this phase transition of ammonium sulfate solution at 1 atm and obtained $\Delta H_{\text{sol}}=48\pm8$ cal g^{-1}.

With the Clausius–Clapeyron equation one finds for the volume alteration of the mother liquid during the phase transition $\Delta v_{\text{sol}}=0{\cdot}058\pm0{\cdot}0095$ cm^3 g^{-1}. The volume alteration of the solution is about 65% of the value for pure water. Because the photographs did not show the characteristic lines of high-pressure ice crystals it was assumed that the mother liquid becomes amorphous in the unit cell of the crystal. Our considerations are based on the assumption that the thermal behaviours of ice crystals and of ice in an amorphous state under the conditions of our experiments are not different in principle. A detailed description of the whole process cannot be given at the present time.

A more quantitative result concerning the freezing damage of the Mb crystals has been obtained by comparing the reflexion intensities of a crystal in the frozen and unfrozen states. The free parameters in a least-square fitting procedure were (1) the change of the conventional X-ray temperature factor *B* and (2) an overall intensity scaling factor. Calculations have shown that the scaling factor remains nearly 1 if the intensities of one frozen crystal are scaled to the intensities obtained from the same sample before cooling. This again shows the absence of appreciable damage. Also *B* varies only slightly (±1 $Å^2$). This was within the limit of our present accuracy. The last result can be used only as an indication of the absence of large statistical displacements of the molecules on a microscopic scale.

It should be noted that the frozen samples showed a number of reflexions with drastically changed intensities. This may reflect the change of the molecular structure due to the high pressure. This change persists even at atmospheric pressure, since at low temperatures the high-pressure phase remains metastable. More accurate statements must await a difference–Fourier analysis. If at low temperatures the lattice remains unperturbed, the *B* value should decrease with temperature. One can take a rotating-crystal photograph to obtain the *B* value from a Wilson (1942) plot, in order to arrive at a quantitative comparison with γ-resonance data of Parak & Formanek (1971). Such experiments are in progress.

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