High-Pressure Freezing Of Protein Crystals.

Anja Burkhardt,[a] Martin Warmer,[b] Saravanan Panneerselvam,[a] Armin Wagner,[c] Athina Zouni,[d] Carina Glöckner,[d] Rudolph Reimer,[b] Heinrich Hohenberg,[b] and Alke Meents [a]

[a] Deutsches Elektronen-Synchrotron DESY, HASYLAB, Notkestraße 85, 22607 Hamburg
[b] Heinrich-Pette-Institute for Experimental Virology, Martinistraße 52, 20251 Hamburg
[c] Diamond Light Source Ltd, Harwell Science and Innovation Campus, Didcot, Oxfordshire, OX11 0DE
[d] Max-Volmer-Laboratory for Biophysical Chemistry and Biochemistry, Technical University Berlin, Straße des 17. Juni 135, 10623 Berlin

Motivation

Protein crystals can contain up to 90% of solvent (mainly water). Cryocooling of such crystals therefore requires the use of cryoprotectants, such as ethylene glycol or glycerol, in order to suppress crystalline ice formation and to convert the water to amorphous ice (vitrification). Finding optimal cryoconditions can be very time-consuming, especially in case of complex systems, such as membrane proteins. Even if adequate cryoconditions have been found, the crystal quality is often degraded upon conventional flash-cooling. Thus, there is a big demand for alternative cryocooling techniques in macromolecular crystallography (MX). A promising approach which allows sample vitrification without cryoprotectants is high-pressure freezing (HPF). This method is well established in the field of electron microscopy for vitrification of cells or tissue.[1, 2] In MX, HPF has been applied to many different proteins of medium molecular weight and unit cell size [3, 4] but successful HPF on large unit cell systems with weak crystal contacts has not been reported to date.

Goal

Our work aims to make HPF an attractive cryocooling tool for crystals which are sensitive to osmotic shock (*e.g.* membrane proteins or viruses) and thus are difficult or even impossible to cryoprotect.

High-Pressure Freezing Experiment

HPF was carried out on three systems: hen egg-white lysozyme (HEWL), cubic porcine insulin and the membrane protein photosystem II (PSII). Protein crystals can either be grown or soaked into thin-walled quartz capillaries and are high-pressure frozen in their mother liquor (*Method A*).[5] The capillaries are cut into 2 mm long segments while submerged under 1-hexadecene. Alternatively, crystals can be directly high-pressure frozen in a drop of crystallization buffer (*Method B*). The samples are sandwiched between an aluminum platelet and a lid and are high-pressure frozen at 210 MPa and 77 K using a Bal-Tec HPM 010 instrument. Sample mounting after HPF is carried out at cryogenic temperatures.

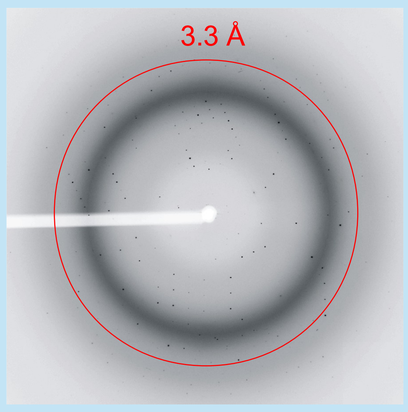
Results and Discussion

> Due to the high cooling rates (7000 K/s) during HPF, both, the solvent inside the crystals as well as the surrounding growth solution, is completely converted to amorphous ice without any cryoprotectants.

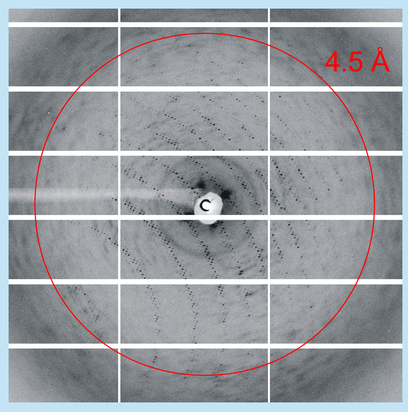
> The diffraction quality of the HPF HEWL and cubic insulin crystals is similar to that of cryoprotected, flash-cooled crystals. In case of HPF cubic insulin an excellent crystal mosaicity of 0.09° is achieved. This makes the method very interesting for large unit cell systems where reflection overlap due to a high crystal mosaicity becomes a problem.

> The applied HPF protocol induces only small structural changes which are comparable to those observed for conventional flash-cooling.

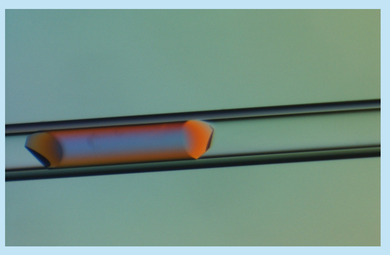
> HPF PSII crystals diffract to ~4.5 Å and show a crystal mosaicity of 0.2 to 0.3° without any cryoprotection.

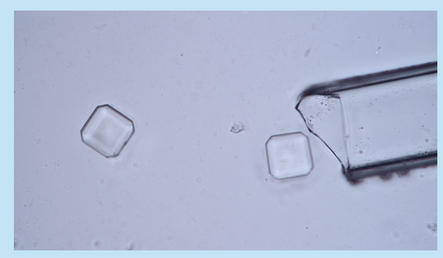


Diffraction images of non-cryoprotected cubic insulin crystals. The HPF crystal (left) diffracts to high resolution (~1.87 Å) and no ice rings could be observed in the diffraction image. The flash-cooled sample (right) diffracts only poorly (~4.8 Å) and diffraction ice rings due to hexagonal ice (indicated by blue arrows) are observed in the diffraction image.



Diffraction image of a HPF PSII crystal. The crystal is not cryo-protected and shows Bragg reflections down to ~4.5 Å.

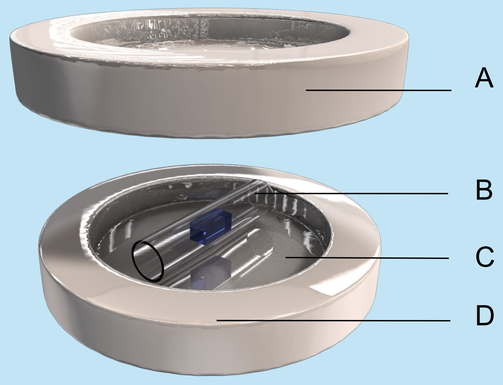




HEWL crystal directly grown in a quartz capillary *via* counter diffusion (left). Transfer of cubic insulin crystals from a hanging drop to a quartz capillary (right).

Bal-Tec HPM 010 high-pressure freezing device.

Sample assembly in the specimen holder for *Method A*: aluminum lid (A), quartz capillary containing the protein crystal in its mother liquor (B), aluminum platelet (D), filled with 1-hexadecene (C).



Summary

In contrast to other HPF procedures published in the field of macromolecular crystallography, the technique presented here involves fast sample cooling which allows complete sample vitrification. Thus, crystals can be directly high-pressure frozen in their mother liquor without cryoprotection or additional sample manipulation steps, such as oil-coating This HPF protocol is therefore ideally suited for cryocooling large unit cell systems which are usually very sensitive and have weak crystal contacts.

Acknowledgements

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1. H. Hohenberg *et al.*, *J. Microsc.* **175**, 24-43 (1994); 2. D. Studer *et al.*, *J. Microsc.* **179**, 321-332 (1995); 3. U.F. Thomanek *et al.*, *Acta Cryst. A***29**, 263-265 (1973);
4. C.U. Kim *et al.*, *Acta Cryst*. *D***61**, 881-890 (2005); 5. A. Burkhardt *et al.*, *Acta Cryst.* **F68**, 495-500 (2012).