High-Pressure Cooling Of Macromolecular Crystals.

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Motivation

Since protein crystals typically contain between 40 to 60% of solvent, penetrative cryoprotectants are required to suppress crystalline ice formation upon flash-cooling to cryogenic temperatures. Finding optimal cryoconditions can be very time-consuming, especially in case of complex systems with large unit cells, such as membrane proteins. Even if adequate cryoconditions have been found, the crystal quality is often degraded upon conventional flash-cooling. Thus, alternative cryocooling techniques are highly demanded in macromolecular crystallography. A promising approach which allows the formation of amorphous ice without adding penetrative cryoprotectants is high-pressure cooling (HPC). This technique is well established in the field of electron microscopy for vitrification of cells or tissue [1, 2]. HPC was also applied to many protein crystals [3, 4], but for large unit cell systems successful HPC has not been reported yet.

Goal

Our work aims to make HPC an attractive tool for crystals which are sensitive to osmotic shock (*e.g.* membrane proteins or viruses) and therefore difficult or even impossible to cryocool by conventional methods.

High-Pressure Cooling Experiment

HPC was carried out on three test 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 quartz capillaries and are high-pressure cooled in their mother liquor (*Method A*). The quartz capillaries are cut into 2 mm long segments while submerged under 1-hexadecene. Alternatively, crystals can be directly high-pressure cooled in a drop of crystallization buffer (*Method B*). The samples are sandwiched between an aluminum platelet and a lid and are high-pressure cooled at 210 MPa and 77 K using a Bal-Tec HPM 010 instrument. Sample mounting after HPC is carried out at 135 K (*Method A*) or 77 K (*Method B*), respectively.

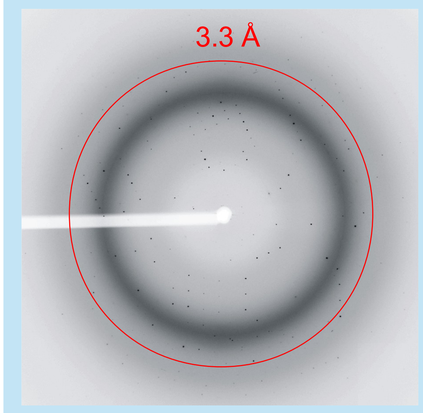
Results and Discussion

> Due to the high cooling rates (5000 K/s) during HPC, the solvent inside the crystals as well as the surrounding solution is completely converted to amorphous ice without the need for any cryoprotectants.

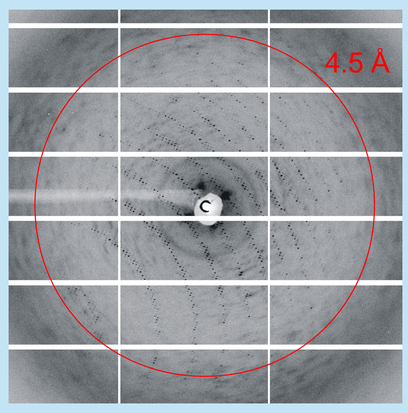
> The diffraction quality of the HPC HEWL and cubic insulin crystals is similar to that of cryoprotected, flash-cooled crystals. In case of HPC cubic insulin an excellent crystal mosaicity of 0.09° is achieved. This makes the method very interesting for cryocooling of large unit cell systems where reflection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

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

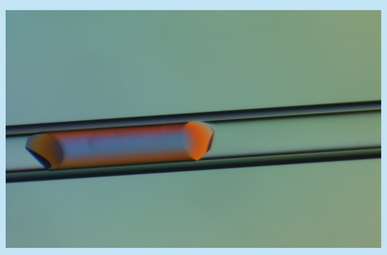
> HPC PSII crystals diffract to ~4.5 Å and show a crystal mosaicity of 0.2 to 0.3° without cryoprotection.

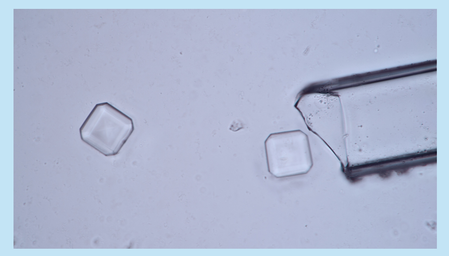


Diffraction images of non-cryoprotected cubic insulin crystals. The HPC crystal (left) diffracts to high resolution (~1.87 Å) and no ice rings could be observed in the diffraction image. The flash-cooled sample (right) diffracts only poorly (~4.8 Å) and diffraction ice rings due to hexagonal ice (indicated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



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

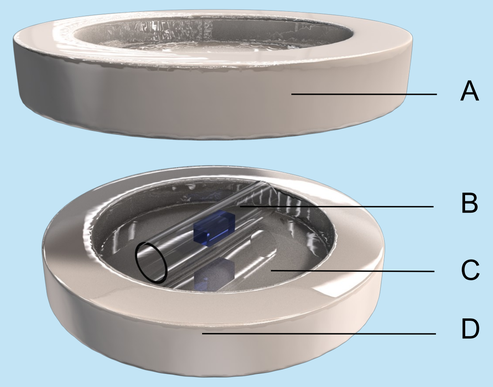




HEWL crystal directly grown in a quartz capillary *via* counter diffusion (left). Transfer of cubic insulin crystals from a hanging drop to a quartz capillary (right).

Bal-Tec HPM 010 high-pressure cooling 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 HPC 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 cooled in their mother liquor without penetrative cryo-protectants or additional sample manipulation steps, such as oil-coating. This HPC protocol is therefore ideally suited for cryocooling of 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).