HPC-201

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HIGH PRESSURE CRYO-COOLER FOR X-RAY CRYSTALLOGRAPHY

...Superior x-ray diffraction patterns

HPC-201 cryocooling was developed as an alternative method for cryopreservation of macromolecular crystals and successfully applied for various technical and scientific studies. The method requires the preservation of crystal hydration as the crystal is pressurized with dry helium gas. Previously, crystal hydration was maintained either by coating crystals with a mineral oil or by enclosing crystals in a capillary which was filled with crystallization mother liquor. These methods are not well suited to weakly diffracting crystals because of the relatively high background scattering from the hydrating materials. HPC-201 is compatible with an alternative method of crystal hydration, called capillary shielding. With this method the specimen is kept hydrated via vapor diffusion in a shielding capillary while it is being pressure cryocooled. After cryocooling, the shielding capillary is removed to reduce background X-ray scattering. It is shown that, compared to previous crystal-hydration methods, the new hydration method produces superior crystal diffraction with little sign of crystal damage. Using the HPC-201, a weakly diffracting protein crystal may be properly pressure cryocooled with little or no addition of external cryoprotectants, and significantly reduced background scattering that can be observed from the resulting sample. Beyond the applications for macromolecular crystallography, it is shown that the HPC-201 has great potential for the preparation of non-crystalline hydrated biological samples for coherent diffraction imaging with future X-ray sources.

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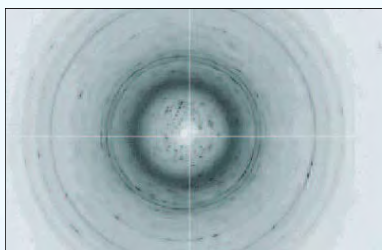
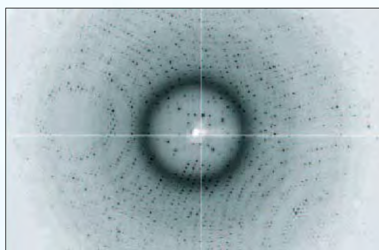
Pharmaceutical companies currently use x-ray crystallography to determine exactly how drug lead compounds and their protein targets interact. To date, x-ray crystallography is the most effective technique in the field of structural biology; out of the approximately 35,000 protein structures solved, x-ray crystallography is responsible for about 29,000. The promise of structural biology to improve human health is great, and any method or device that can speed the solving of protein structures will contribute to fulfilling that promise.

Cornell University developed a novel method to cryo-cool protein crystals without the need for penetrative cryoprotectants. The method involves mounting protein crystals in a cryoloop with a thin coating of oil, pressurizing the crystal up to 200 MPa (2000 atm) in He gas, cooling the crystal under pressure, and then releasing the pressure. This process results in dramatic improvement in diffraction quality in terms of diffraction resolution and crystal mosaicity.

This device's fundamental design is based on a process developed and patented by Cornell University scientists Prof. Sol M. Gruner (http://bigbro.biophys.cornell.edu/) and Dr. Chae Un Kim; US Patent No. 8,030,449. Flash-freezing at atmospheric pressure typically requires the use of cryoprotectants. Finding the right cyroprotectant for each sample type can be a long, trial-and-error process. The High Pressure Cryo-Cooler eliminates the need to use cryoprotectants and produces superior results.

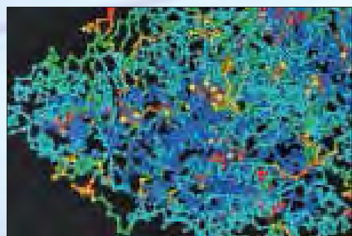




The project was first funded by The National Institutes of Health. The National Institutes of Health (NIH), through its National Institute of General Medical Sciences (NIGMS), funds MacCHESS for two purposes: core research as motivated by important biomedical problems and support to all structural biologists making use of the CHESS facility for crystallographic and small-angle X-ray scattering experiments, as well as for novel experiments requiring special equipment and staff assistance not readily available at other synchrotron sources.

Process

Once a protein crystal has been picked up using a loop, or similar device, the crystal is analyzed using x-rays. Unfortunately, a typical protein crystal at room temperature survives only a fraction of the x-ray dose required for a complete high resolution data set before it becomes irrevocably radiation-damaged. To inhibit the radiation damage, protein crystals are typically flash cooled at atmospheric pressure by plunging them into liquid nitrogen (77 K or -196 °C). Cryocooling also reduces the thermal motion within the crystal, enabling the collection of higher quality data.



Freezing protein crystals successfully and without physical damage, however, is a tricky business. Proteins crystals form in an aqueous solution and can contain 50 percent, or more, water by weight. As anyone knows who is lucky enough to be by a northern lake on a frigid night early in the winter, as the ice noisily heaves and cracks, freezing water expands with great force—more than enough to damage the crystals one is trying to protect. The goal of flash freezing is for the water to form amorphous ice rather than crystalline ice. Cryoprotectants are typically added to promote this result. Unfortunately, since each protein is unique, a specific cryoprotectant must be formulated for each, a task that proves difficult or impossible in many cases.

Professor Sol Gruner, and Dr. Chae Un Kim's innovations have eliminated the need for cryoprotectants in many cases while increasing cryocooled protein crystal quality. Instead of freezing protein crystals at atmospheric pressure, they cryo-cool their protein crystals under high pressure. Under these conditions, the water turns into a higher density form of amorphous ice, which minimizes crystal disruption.

This process is simple in concept—pressurize a protein crystal in helium at room temperature up to between 100 and 400 MPa (about 14,500 to 58,000 psi), cryocool the crystal to 77 K, then release the pressure while maintaining the low temperature—but complicated in practice, due to the high pressures involved.

Protein crystal structure is solved by determining the repetitive three-dimensional electron density distribution of protein molecules in a crystalline arrangement. Each crystal reflection has an intensity (amplitude) and phase, and both are needed to generate a protein structure. The information obtained from an x-ray diffraction experiment provides intensity data directly. In some cases, it is possible to use "anomalous diffraction" to also obtain phases. With single-wavelength anomalous diffraction

(SAD), all the data needed to solve a structure can be obtained from a single crystal. However, anomalous signals are weak, and a good-quality crystal containing some anomalously scattering atoms is essential for successful SAD. Gruner and Kim modified their high pressure cryocooling method for SAD by adding a first pressurization step in krypton or xenon gas, before switching to helium. During this additional step, krypton or xenon atoms bind to specific locations in the protein. By taking advantage of the anomalous signal from these "heavy" atoms, complete phasing information is derived. Kim proved the efficacy of this method by solving the structure of porcine pancreas elastase from a single crystal cryocooled with Cornell's new process. In other cases, the crystal quality was sufficient to allow phasing by native sulfur SAD.

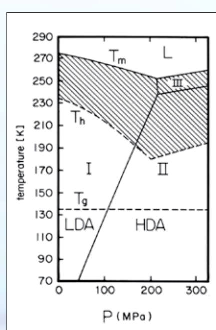
The "High Pressure Cryo-Cooler for X-Ray Crystallography (HPC-201)" provides the proper equipment to make the process practical for structural biologists to use. This system can be configured for interchangeable gas tanks, such as the introduction of Kr or Xenon.

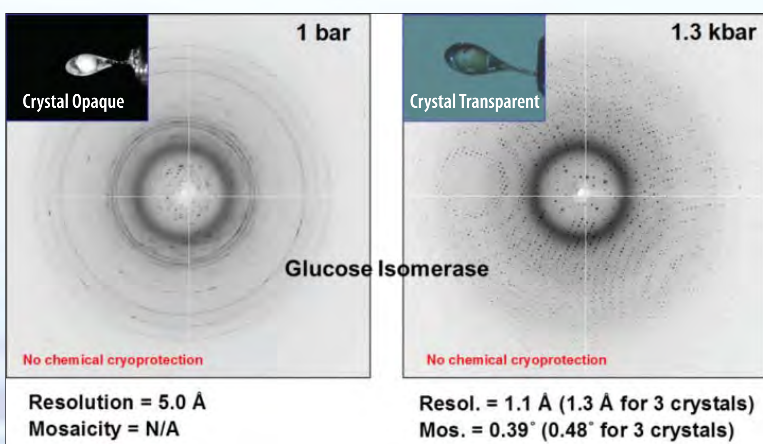
Proposed Mechanism

Cornell University successfully pressure cooled dozens of different proteins. It was observed in almost all cases that water vitrification in protein crystals was achieved without any penetrative cryoprotectants. Furthermore, in many cases, improvement in diffraction quality compared to ambient pressure flash cooling was observed. These effects involve the dynamics of water under pressure and the formation of high density amorphous (HDA) ice.

Water Vitrification

Protein crystals frozen in droplets of mother liquor at room pressure and at 1,300 atmospheres pressure are shown below. The crystalline ice in the photo below is cloudy, while the amorphous ice in the other photo below is transparent. The bottom two images are the x-ray diffraction patterns captured from the corresponding protein crystals. The rings on the pattern on the lower left photo arise from ice crystals. More importantly, the quality of the diffraction obtained by the pressure-frozen crystal (below right) is superior to the diffraction from a crystal frozen at room pressure (below left).





Diffraction Improvement

When protein crystals are cooled at room pressure, the unit cell volume usually shrinks by ~ 5%. But liquid water inside the crystals turns into either ice I or LDA (Low Density Amorphous) ice which expands by ~ 6.7 % (0.94 g/cm3 at 77 K, 0.1 MPa). This volume discrepancy leads to disorder in the crystal. When rapidly cooled at high pressure, however, liquid water may freeze into HDA (High Density Amorphous) ice and, once formed, it stays metastable in the HDA state when the pressure is removed, as long as the temperature is kept below 120 K. HDA ice has higher density (1.17 g/cm3 at 77 K, 0.1 MPa) and this mitigates the volume related crystal disruption.

HPC-201 Specification

The high-pressure cryo-cooler is designed to hold 3 samples at a time. Crystal samples are picked up using a standard cryoloop. Cryoloops are mounted into heavy duty stainless steel tubing in the unit and are then ready to be pressurized and cooled. A high pressure oil pump provides helium gas to the samples. External controls allow the sample to be first pressurized and then cooled in an LN2 bath. Once pressure is released the samples can be removed and handled like any other samples prepared by conventional flash freezing.

TABLE OF FEATURES:

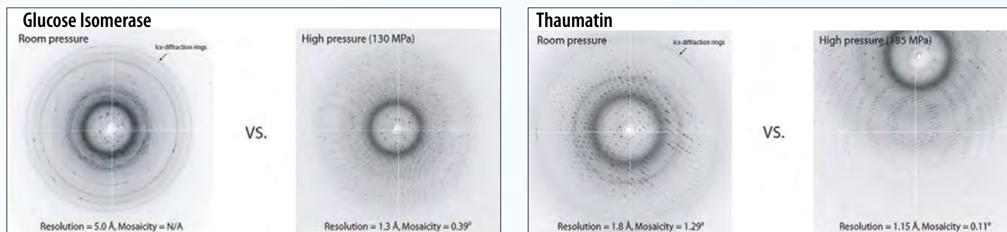
Pressurizing Gas	Helium or compatible inert gas
Working Pressure	Up to 200 MPa
Cooling Fluid	LN_2
Cryo Cooling Temp	77 K (-196 °C)
Sample Capacity (per pressure & cooling cycle)	3
Process Time	< 10 min (2 min for pump operation; ~5 min under pressure; 1 min freezing)
ZEISS Microscope	SteREO Discovery. V8
LN2 Dewar	Taylor-Wharton HC34

TABLE OF PROPERTIES:

Voltages, frequencies	50/60 Hz
Power input, approximately	100 to 230 VAC
Oil pump pressure	200 MPa
LN_2 Dewar holding	200 Days

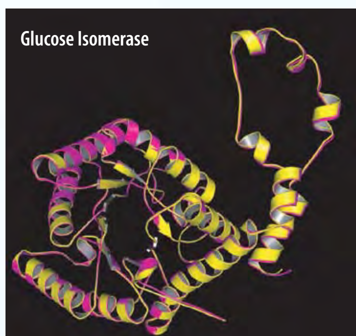
Experimental Results

Two cases with dramatic improvement in diffraction quality are shown below for glucose isomerase and thaumatin.
(Contribution from: Chae Un Kim & Sol M. Gruner; Cornell University)



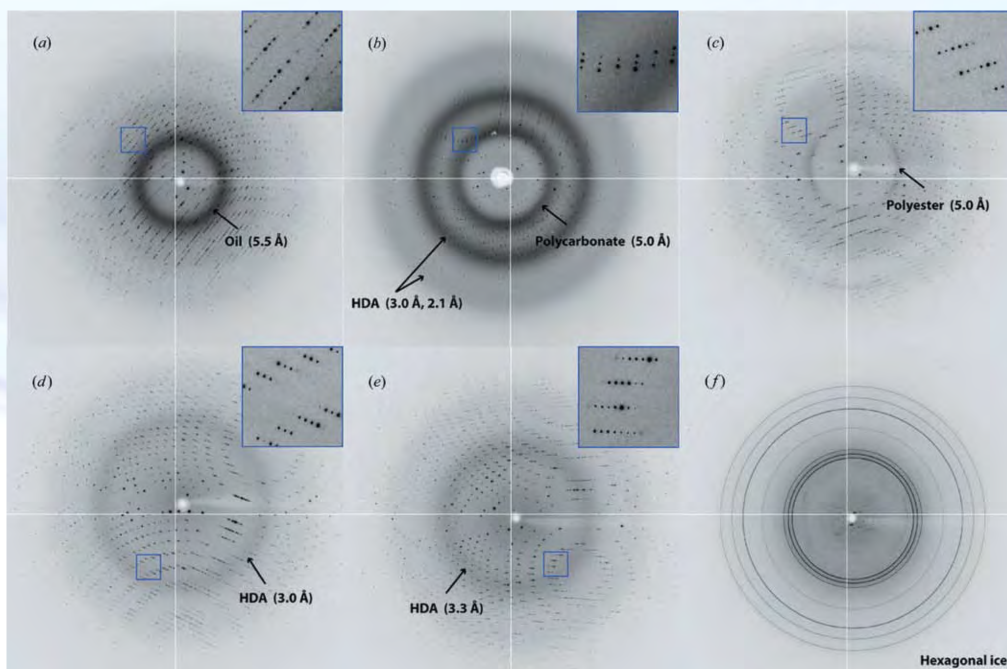
Protein Structure Comparison

The difference (r.m.s. deviation) between the room pressure flash-cooled structure and the pressure cooled structure is less than 0.5 Å in both cases, which is comparable to the differences seen when changing temperature.



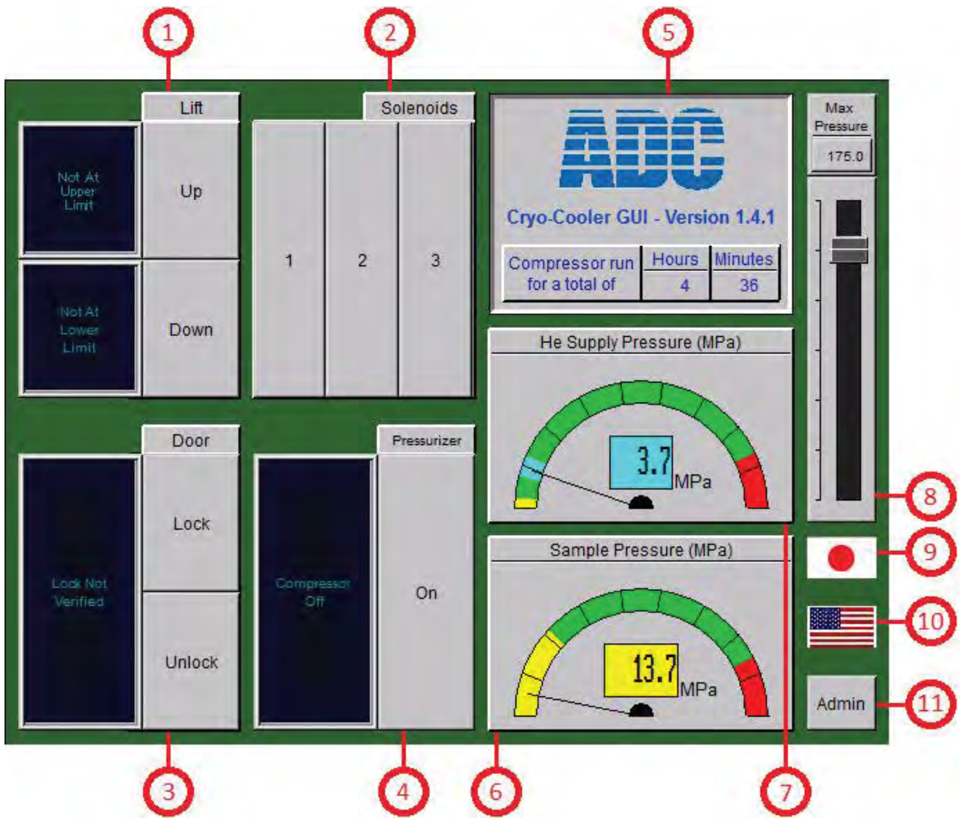
Yellow: PDB code 8XIA, room pressure, 288 K Magenta: high pressure (130 MPa), 110 K

Yellow: PDB code 1LXZ, room pressure, 110 K Magenta: high pressure (185 MPa), 110 K



The above picture references diffraction images of high-pressure cryocooled crystals prepared by three hydration methods. (a) Oil-coating method (Fig. 1a). The image shows diffuse scattering at around 5.5 Å from oil. Crystal mosaicity is 0.67°. (b) Capillary-hydration method (Fig. 1b). The innermost diffuse ring (~5.0 Å) is generated from the polycarbonate capillary, and the second and third diffuse rings are from the solvent (HDA ice) inside the capillary. Crystal mosaicity is 0.34°. (c) Capillary-shielding method, but without removing the polyester capillary (Fig. 1d). Although the scattering from the polyester capillary is present (anisotropic peak at ~5.0 Å), the background level is dramatically reduced compared to the oil-coating and capillary-hydration methods. Crystal mosaicity is 0.11°. (d) Capillary shielding with removal of the polyester capillary (Fig. 1e). The image shows faint diffuse scattering around 3.0 Å, which is from the small amount of external and internal solvent (HDA ice) in and around the crystal. Note that the level of diffuse scattering from the solvent is comparable to the air scattering around the beam stop. Crystal mosaicity is 0.12°. The slight increase in unit-cell dimensions and mosaicity from 0.11° is due to X-ray radiation damage (Ravelli & McSweeney, 2000). (e) Crystal equilibrated to 10% (v/v) glycerol in deionized water (Fig. 1f), then prepared by the capillary-shielding method; the polyester capillary was removed before data collection. The diffuse scattering from the solvent (HAD ice) is shifted to 3.3 Å owing to the addition of glycerol and removal of NaK tartrate. Crystal mosaicity is 0.08°. (f) Diffraction image of the crystal after warming to room temperature and being refrozen to 100 K in the N2 gas cold stream at the beamline at ambient pressure (Fig. 1g). It shows strong diffraction rings from hexagonal ice formation and very poor quality diffraction from the protein crystal. The result indicates that the crystal was kept hydrated during the capillary-shielding method. (From *Journal of Applied Crystallography*; Chae Un Kim, Jennifer L. Wierman, Richard Gillilan, Enju Limac and Sol M. Gruner; 2013 46, 234-241)

HPC-201 Control Panel:



1) **Lift control** – This controls the lift movement. While the lift is moving, one of the buttons will not be visible and the other will be turned into a stop button. The blue indicators will brighten when the lift is at a limit switch. The buttons will not be available if a solenoid is being triggered.
2) **Solenoid control** – This control will activate the solenoids, removing the magnetic holder to release the sample tube. Only one solenoid can be active at a time.
3) **Door control** – This control locks and unlocks the door. The door handle must be in the upright position and then the lock button must be pressed to properly lock the door. The blue indicator is wired to a confirmation signal from the lock itself. Note that the door cannot be unlocked if the compressor is on or the sample pressure is above 1 MPa.
4) **Compressor control** – This control activates and deactivates the compressor. This button will not work unless the indicator in the door control is lit. The indicator will flash yellow and blue when the pressurizer is on.
5) **Information panel** – This has a compressor total run time counter for maintenance purposes. This total can be reset in admin mode. This is also the location where errors and warnings appear if there is one.
6) **Sample pressure meter** – This is the readout of the pressure from the output of the compressor and the pressure that the sample will be exposed to. The screen will turn red when the pressure is above 50 MPa as a warning. The red location on the bar is set to the maximum pressure and the compressor will not go above it, this can be adjusted with the maximum pressure slider (number 8).
7) **Helium supply pressure meter** – This measures the input pressure of the compressor. The recommended pressure is designated by the cyan region which is 3 MPa through 5 MPa
8) **Maximum pressure slider** – This sets the pressure the pump will compress to before stopping. The range available is between 50 MPa and 200 MPa. If the button above the slider is pressed an exact amount can be entered.
9) **Language Control (Japanese)** – This button translates the language of the screen to the national language that the flag represents
10) **Language Control (English)** – This button translates the language of the screen to the national language that the flag represents
11) **Admin Screen** – This button is used to go to the admin screen after entering a passcode. The admin screen can be used to override safety features such as running the compressor with the door open. This is also where you can reset the compressor run total. Please contact the factory for the passcode.

For more information on ADC's HPC-201, including a short video please visit us at:
http://www.adc9001.com/products/view/455

For more information regarding the publications used in this brochure please visit us at:
http://www.adc9001.com/data/Cryo_Cooler_Paper_links.pdf

HPC-201
Unique Features:

- Automatic freezing of samples with one button operation
- Perfect freezing of samples up to 3 mm cryoloop diameter
- No cryoprotectants required
- Rapid transfer loading device
- Graphical data display of real time pressure for each run
- Integrated work station
- Integrated high-end PLC with touch screen interface
- Universal and application specific sample carriers
- Integrated Dewar with drain outlet
- Safety process chamber
- Multilingual operating (German, French, Spanish, Chinese, Japanese, etc.)
- Integrated video camera
- Freezing process cover for ultimate safety
- Integrated 200 MPa compressor
- Compact and mobile
- Uses standard 100–250 V / 50-60hz power supply