

Shock freezing of macromolecular crystals at MacCHESS

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The collection of macromolecular diffraction data has recently benefited from technique and equipment advances which have made low temperature data collection almost routine. The ability to freeze protein crystals at liquid nitrogen temperatures normally gives the level of radiation protection necessary to collect complete data sets from single crystals. The need to improve the radiation sensitivity of protein samples is an even more key issue at such intense x-ray sources as CHESS. To this end, MacCHESS has upgraded both its equipment and its capability to offer support to assure that visiting user groups can routinely collect low temperature data while at the synchrotron.

The collection of low temperature data on protein samples hinges around a loop mounting technique first developed here at Cornell by T.Y. Teng. In the original technique, crystals were mounted by lifting them in thin wire loops by surface tension from soaks of mother liquor containing some cryoprotecting molecule. These molecules were generally freezing point depressing solutes such as sucrose or low molecular weight alcohols such as glycerol, ethylene glycol, isopropanol, or methylpentanediol (MPD). In addition to such alcohols, lower molecular weight polyethylene glycols such as PEG 400 or 600 have been used successfully. Recently, the technique has been improved by replacing the wire loops with loops fashioned from glass wool, rayon, nylon, silk, or any other thin, non-diffracting fiber. Using such equipment, 360° of continuous rotation data can be collected from a single crystal without worrying about significant shadowing or increases in absorbance or scatter from the loop mounts. A schematic of a loop design currently used at CHESS and a photograph of an actual

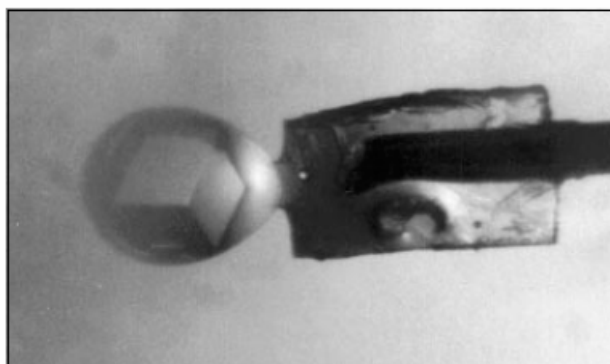
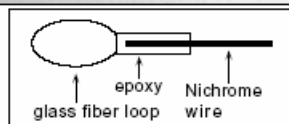


Figure 1. (Above) Freezing-loop-mounted crystal of nucleoside deoxyribosyl transferase (crystal dimensions are approximately 600 x 600 x 400 microns²) within a drop of cryoprotectant fluid. (Right) Schematic of the crystal mounting loop.



loop mounted crystal are shown in Figure 1.

The key to shock freezing crystals is maximizing the efficiency of the initial freezing rate, or the shock. Two methods are currently used to accomplish this. First, crystals can be frozen directly in the cold stream. In this method, a reliable cryostat capable of maintaining a laminar cold stream at -165°C to -175°C with a good flow rate is essential. Actual freezing involves temporarily deflecting the cold stream with something as simple as an index card while the crystal is quickly seated in the diffraction position (usually using a goniometer mounted magnet and a loop attached to a steel mounting cap). Then, the stream deflection is removed and the low temperature gas is used to flash freeze the crystal. In the second technique, loop mounted crystals are first immersed in some low temperature liquid such as liquid nitrogen, propane, or freon. Liquid propane freezing is the most

likely trend of the future as it provides the best thermal capabilities for heat conductance away from the immersed crystal. After immersion, the crystal is seated at the sample position still in the low temperature liquid, and the vessel containing the liquid is quickly removed. In this case, the liquid is used to cause the initial flash and the challenge is smoothly transferring the frozen crystal to the gaseous cold stream. Both techniques have been used successfully at CHESS by outside and local user groups.

As has been mentioned, equally important as technique to crystal freezing is the availability of good cryostats. There are two different cryostat systems available to shock freeze protein crystals at MacCHESS. The newest and most reliable units are the heat exchange MSC devices (Molecular Structure Corporation, The Woodlands, TX). Figure 2 shows a schematic of an MSC cryostat. A 160 liter Taylor-Wharton XL-45 liq-

uid / gas dewar (or more recently, a 240 liter Cryofab CLG-240-PB-C dewar) serves as the dry nitrogen gas supply. The gas pressure at the outlet is regulated to 25 PSI and fanned out to two gas lines, both equipped with flow meters and needle valves for fine control. One line is fed through a copper coil inside the 100 liter stainless steel wide mouth dewar which serves as the heat exchange reservoir. The gas reaches equilibrium temperatures near 77K in the coil and is transferred to the nozzle, placed within 1 cm of the sample position, by an evacuated stainless steel transfer tube. The second line is fed directly into an outer jacket around the nozzle and produces a collinear warm air outer stream which sheaths the inner stream from turbulence and prevents freezing of the nozzle. A heater and a thermocouple contained upstream of the nozzle give the ability to maintain and monitor any given temperature from +30°C to -190°C within ±1°C. Several transfer lines which provide the option of spindle collinear or perpendicular cold-streams are available at MacCHESS.

As an alternative, the older boil-off "MAX Cryostat" system developed at the Max-Planck Research Unit for Structural and Molecular Biology (Hamburg, Germany) is avail-

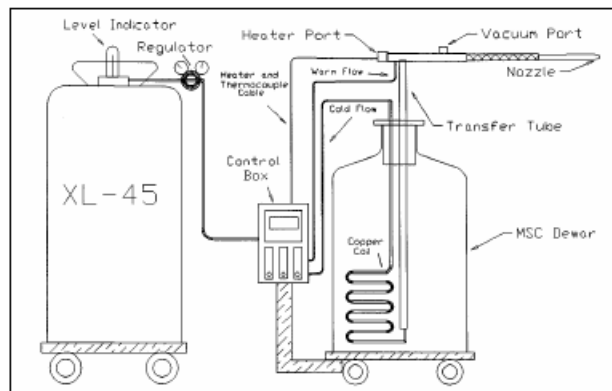


Figure 2. Schematic of Molecular Structure Corporation (MSC) cryostat and supply dewar.

able upon request and as an emergency backup. This system provides a stable temperature of better than -160°C at the sample, but suffers from a rather poor flow rate which can be problematic in difficult crystallographic problems if stream freezing is to be done. Low temperatures are achieved by using boiled off gaseous nitrogen supplied by the smaller evaporator dewar. A slight overpressure is produced inside the evaporator by an electrical resistor immersed in the liquid nitrogen. The

reservoir of nitrogen in a larger supply dewar is used to maintain a constant level of liquid nitrogen in the evaporator using an automatic level control device. When a platinum thermometer (PT100) inside the evaporator signals a low level, an electrical resistor at the bottom of the transfer dewar heats, boiling off nitrogen and forcing liquid nitrogen through a transfer line into the evaporator. While more fragile due to vacuum glass gas transfer lines, less reliable due to questionable electronics, and not capable of the high flow rate achievable with the MSC units, it does serve as a competent backup for emergency situations. Both systems provide the option of continuous refill allowing indefinitely long experiments with no fluctuation in sample position temperature.

Using the available equipment resources at CHESS, almost all protein data now collected are at low temperature. In fact, the beamline upgrades on the new A1 station have made it virtually impossible to collect anything but low temperature data without beam attenuation. In the unattenuated beam at A1, lysozyme crystals last for only 90-120 seconds of exposure at room temperature (previously, multiple data sets could be collected from single crystals), while even radiation sensitive

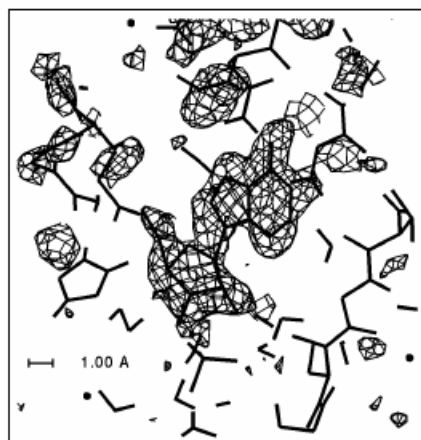


Figure 3. Section of a difference Fourier map of a complex between the enzyme bovine purine nucleoside phosphorylase and 9-deaza-inosine, a noncleavable substrate analogue. A refined model of the substrate analogue and the protein is shown fit to the difference density. All density not modelled by the substrate analogue is associated with a loop to helix conformational change in the protein induced by substrate binding. The map was calculated with the program PHASES using $\rho_{\text{difference}} = \rho_{\text{complex}} - F_{\text{protein}} \rho_{\text{substrate}}$ as coefficients.

samples can last almost indefinitely at low temperature. As evidence, a high resolution data set on a radiation sensitive crystal form in this lab (nucleoside deoxyribosyl transferase) which previously required 43 crystals at room temperature can now be done using a single crystal at low temperature. Similar successes have been achieved with almost every crystal attempted. High quality data at resolutions extending to 1.0Å have been collected on frozen crystals in the past months as shown by the 1.4Å data with an overall R_{sym} of ~3% collected on a single frozen crystal of lipoygenase provided by Wladek Minor of Purdue University. Further, concerns of increased mosa-

icity and irreproducibility of unit cell constants from crystal to crystal have not been a problem in any project collected in the past six months.

As an example, figure 3 shows that good difference Fourier maps have been calculated using native and complex data collected at CHESS from frozen crystals of bovine and human purine nucleoside phosphorylase, showing a lack of crystal variation upon freezing. Thus, most concerns relating to low temperature data collection have been overcome and it is becoming almost a standard protocol in MacCHESS diffraction experiments.

In conclusion, MacCHESS now provides the resources necessary for

routine low temperature data collection on macromolecular samples. In addition to providing good, reliable, commercial cryostats, a level of expertise has been developed locally from interaction with visiting user groups at the forefront of development in this area. MacCHESS is currently planning a video loaning library in which the initial volume will discuss and demonstrate the techniques and theory of low temperature crystallography. In the interim, local support will gladly be provided at any level by contacting Rick Walter in the laboratory of Dr. Steven Ealick (email address: walter@vgx.tn.comell.edu).