Summary of lecture and practical session at Biophysics and Structural Biology at Synchrotrons workshop: cryo-cooling in macromolecular crystallography—why and how?

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A pivotal step in the usual macromolecular crystallographic structure solution pipeline is the cryo-protection and cooling of crystals for data collection at temperatures of around 100 K. About 80% of structures currently in the PDB were derived from diffraction data collected at or near 100 K, since the rate of radiation damage to the crystal is reduced in general by around a factor of 70 compared with collection at room temperature (RT). Radiation damage during the experiment manifests as a reduction in diffraction resolution, increased scaling B-factors, and expansion of the unit cell (termed global damage, detected in reciprocal space) and also in the breaking of disulphide bonds and decarboxylation of aspartate and glutamate residues in a well-defined and reproducible order (specific damage in real space). When 12.4-keV X-rays are incident on a 100- μ m-thick metal-free crystal, about 2% of them interact with the atoms (solvent and protein) and of these, about 8% are elastically scattered and give the diffraction pattern we seek. The remaining 92% of interactions involve absorption of the X-rays, and the energy thus deposited in the crystal induces ionisation and the formation free radical species. At RT, these radicals are mobile and cause further damage to the crystalline order in a domino-type effect, whereas at 100 K, most species are immobilised. However, even at cryo-temperatures, electrons can quantum mechanically tunnel to

the most electron-affinic sites, and holes are also mobile, leading to site-specific damage.

Apart from significantly reducing the rate of radiation damage, cryo-cooling has additional advantages: usually a complete dataset can be collected from a single crystal (so fewer crystals are required and increased systematic errors caused by merging data from different crystals are avoided), if using multi-wavelength anomalous dispersion phasing methods, systematic errors are minimised by using only one crystal, crystals can be harvested and stored while they are in peak condition, small crystals and those that grow as flat plates can be mounted easily in cryo-loops, there is no evidence for secondary radiation damage progression if crystals are stored under liquid nitrogen having first been irradiated, and a range of new experiments is possible (e.g. soaking in substrate and cooling at different soak times to follow the reaction intermediates).

How can we cool the crystal to 100 K without destroying the ordered lattice? Small changes in procedure can have a significant impact on the resulting resolution of the data and thus impact the quality of the biological information obtained. The crystal must be cooled so fast that the solvent in the intermolecular cavities becomes amorphous glassy solid, rather than forming ordered ice crystallites. The latter disrupt the internal ordered lattice when they form as their volume is greater than that of liquid, and also give powder diffraction rings that obscure reflections at particular resolutions (3 intense powder rings between 3.5 and 3.9 Å). Thus, it is often necessary to add an organic solvent, a ‘cryo-protectant’ such as glycerol, ethylene glycol, or light polyethylene glycols to the crystal buffer and soak the crystal in it prior to swiftly plunging it into liquid nitrogen (77 K).

Thus optimising the cryo-protection step is important for the final outcome, and typically, the experimenter takes the crystallisation buffer and dilutes it with the cryo-protectant.

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Sometimes the crystal (imagine YOU are the crystal!) does not take kindly to this treatment, since the protein solubility might increase and then the sharp edges of the crystal start to become rounded, or at the other extreme, the osmotic pressure of this cryo-protectant buffer might be much larger/smaller than prior to soaking, and small ‘crazy paving’ cracks will appear on the crystal surface. If either of these problems is encountered, it is worth making up the cryo-protectant buffer by replacing the water in the original crystallisation buffer with the cryo-protectant agent (i.e. making up the latter at double concentration and then adding water and cryo-protectant agent to reproduce the original crystallisation buffer concentrations). In problematic cases, this method has often given very good results. Also, if osmotic shock is the issue, sequential *in situ* soaking in increasing concentrations of cryo-protectant buffer can have a huge positive impact (e.g. the resolution of subtype N6 influenza neuraminidase crystals, which required 40% glycerol for optimum protection, routinely increased from 3.2- to 1.6-Å resolution after using this protocol).

For two reasons, harvesting just one crystal at a time for soaking in the cryo-buffer is recommended, rather than pipetting the buffer onto a whole drop containing many crystals. Firstly, many cryo-protectant agents cause dehydration and shrinkage of the unit cell, and since fishing them takes time for each crystal, they will spend varying times soaking and thus have a range of unit cells, which will give increased systematic errors if data from different crystals is to be merged. Secondly, many crystals will remain stable in the cryo-protectant buffer for a limited time, but will then start to degrade, so it is unwise to leave them in it for too long while cooling the other crystals.

The next step is to ‘fish’ the crystal into a cryo-loop under a microscope and then plunge-cool it into a Dewar of liquid nitrogen, first checking that there is no skin over the drop and that it is not stuck to the bottom of the well by stirring the drop with a tool (acupuncture needle, cat’s whisker, etc.). To minimise the amount of liquid round the crystal (and thus minimise the background X-ray scattering), the loop can be brought out of the drop with its plane perpendicular to the drop surface. To avoid ice forming in the liquid nitrogen, the Dewar should be kept as full as possible and have an

easily removable lid on top of it during ‘fishing’, and the nitrogen should be regularly renewed.

If the cryo-cooled crystal does not diffract, testing at RT can be very worthwhile as then it can be determined whether the crystal lattice was destroyed by the cryo-cooling protocol which should then be altered (soak time, soak temperature, cryo-protectant) or whether it was never well ordered in the first place, and new crystallisation trials are then required.

Crystals for data collection at 100 K at synchrotrons are typically now cooled into the pucks that fit straight into the beamline mounting robot, and then shipped to the synchrotron in special transport Dewars. These Dewars should be allowed to dry out after every trip, in order to disperse water vapour that has condensed and been trapped inside when they are opened and closed.

During the 1-h crystal mounting practical session at the Workshop, 8 separate groups of 6 students each successively practised preparation for both RT and cryo-cooled experiments, with all students carrying out every exercise. Following the demonstration of the MiTeGen RT mounting system, they (see photo below) practised manipulating the polyamide sleeve (which is sealed at one end and contains a small volume of the crystallisation buffer which has been previously pipetted into the end of the sleeve) over the cryo-loop using the guide groove former. This prevents crystal dehydration during RT data collection and can be a tricky operation. Next, students practised the actions involved in flash-cooling a crystal into liquid nitrogen, including keeping the crystal moving gently after plunging it, in order to leave behind gaseous bubbles of nitrogen which insulate the crystal from the cold liquid and which can prevent efficient cooling. They also practised feeding the crystal pin straight into a puck by looking directly down the wand held perpendicularly to the plane of the puck (i.e. putting their faces over the Dewar while wearing safety glasses to line up the pin carefully). Finally, all students successfully managed to ‘fish’ a crystal into a cryo-loop under a microscope which also had a video feed so that everyone in the group could watch the manipulations and cheer them on!

Crystal mounting practical. Left to right: the author, Kesolofetse Kehiloe (University of Cape Town, RSA), Natalia Galdi Quel (UNICAMP, Brazil). Kesolofetse is practising crystal mounting for RT data collection using the MiTeGen system. She is placing a plastic sleeve over a crystal mounted in a cryo-loop, using a sleeve guide so that the crystal is not

deposited on the inside of the sleeve instead of staying in the loop. The size of the loop has been matched to the size of the crystal before ‘fishing’ so that the crystal cannot move around in the liquid across the loop during the RT data collection.

Compliance with ethical standards

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Conflict of interest Elspeth F. Garman declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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