SOAKING, MOUNTING, AND FREEZING PROTEIN CRYSTALS

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Most X-ray crystallographic data collection is done at low temperature (typically 100 K) to minimize degradation of the crystal by free radicals generated by the X-ray beam. This is especially important when using intense synchrotron X-ray sources. In order to prevent crystals from cracking when frozen, it is necessary to treat protein crystals with a cryoprotectant prior to freezing. In the presence of a cryoprotectant, the protein and its thin layer of surrounding mother liquor will form an amorphous glass in which the crystal suffers minimal damage, and retains maximum X-ray diffraction properties.

SCREENING FOR A SUITABLE CRYOPROTECTANT

Unless the optimum crystallization conditions already contain a sufficient quantity of cryoprotectant, it will be necessary to experimentally determine solution conditions suitable for safely freezing crystals. Typically, some quantity of cryoprotectant is added to a solution of artificial mother liquor, or a solution of artificial mother liquor containing the appropriate amount of cryoprotectant is made up from scratch. Some typical cryoprotectants and concentrations required to assure proper freezing protection in the worst-case scenarios is given in Table 1 below. In many cases a lower concentration of cryoprotectant that that listed in Table 1 is sufficient. (For example, crystallization solutions already containing high concentrations of PEG may require little or no additional cryoprotection.) The minimum amount of cryoprotectant required can be determined by taking up a small quantity of liquid in a crystal mounting loop and plunging it into liquid nitrogen. If drops reliably freeze clear, then the solution has sufficient cryoprotection for freezing protein crystals.

The choice of cryoprotectant will depend upon the crystallization solution composition. If protein crystallization conditions already contain a cryoprotectant, it is often ideal to simply increase the concentration to the appropriate value. This is especially convenient for PEG-containing solutions. However, PEGs have limited solubility in solutions that contain high concentrations of salt; in this case one of the other cryoprotectants in Table 1 is more likely to be suitable. Glycerol, glucose, or sucrose are very gentle to most proteins, have high solubility in a large variety of solution, and are often excellent choices.

Typical Cryoprotecants and Concentrations Required

- glycerol, 30% v/v
- sucrose, 30% w/v
- glucose, 30% w/v
- ethylene glycol, 30% v/v
- MPD, 30% v/v
- PEG-400-2000, 25-40% v/v or w/v

Once a suitable cryoprotectant solution or solutions have been identified, the behavior of protein crystals in these solutions should be observed. This is often carried out at the same time as crystal mounting, as described below. You should observe that the crystal does not disintegrate, crack, or split during cryo-soaking. It is not necessary to soak crystals for extended periods to confer cryoprotection. All that is necessary is to replace the solution on the surface of the crystal with the cryoprotectant solution, a process that only takes a few seconds of soaking. For crystals that do not survive direct transfer into cryoprotectant solutions, try the "No-Fail" method of cryoprotection, described below.

MOUNTING PROTEIN CRYSTALS ON LOOPS

Protein crystals are mounted for diffraction on tiny nylon loops 0.05–1.0 mm in diameter. The loops are mounted on hollow rods that are in turn mounted on magnetic caps that are conveniently stored under liquid nitrogen, and are easily placed on the goniometer head of the X-ray diffractometer. A photo of a loop and cap is shown below (Figure 1).

Figure 1. Mounting loops and cryovials

The following protocol is typical:

- Obtain and don comfortable Thinsulate gloves to protect your hands from frostbite.
- Fill a tall dewar with liquid nitrogen, and insert and cool a labeled cryo-cane to hold your mounted crystal samples. Fill a second dewar with liquid nitrogen to periodically top off the first dewar.
- Obtain a vial clamp and a crystal wand for handling vials and crystal caps.
- Obtain a collection of cryovials fitted with crystal caps with various sizes of mounting loops. The caps are color coded to aid in indentification.
- Obtain your crystal tray containing crystals to soak, freeze, and mount.
- Obtain a spot plate, a 20 µL pipettor and pipette tipes, and your cryoprotectant solutions.
- Assemble all of these materials around the dissecting microscope.
- Place the crystal tray under the microscope and focus on a well containing suitable crystals. Without removing the coverslip, determine what size loops are appropriate by holding them under the microscope next to the coverslip. You should choose a loop size that is just slightly larger than the crystals.
- Pipette 10-20 µL of cryoprotectant in a spot plate well
- Label[1] a cryovial bottom and mount it in the vial clamp
- For the next steps you must work quickly, as the protein drop may evaporate rapidly, causing protein precipitation or crystal cracking.[2]
- Carefully remove the coverslip with the desired crystals and place it drop-side up over an empty well of the spot plate.
- Mount an appropriate size loop on the crystal wand and fish out a crystal. The loop should be just larger than the crystal. If you maneuver the crystal close to the edge of the drop it will be easier to pick up.
- Place the crystal into the cryoprotectant solution by touching the loop to the drop.
- Observe the crystal under the microscope to check for cracking or disintegration. It is not necessary to soak the crys ⓘ for more than a few seconds in order to confer cryoprotection. If there are no problems, fish out the crystal in the loop and

immediately plunge it rapidly into liquid nitrogen and keep it there. If the crystals crack or disintegrate, you need to find another cryo-soak.

- Immerse the empty cryovial into the liquid nitrogen until it stops bubbling. Keep both the crystal cap and the vial under the surface, and screw the crystal cap into the cryovial and mount the vial in the cane.
- Mount additional crystals as required before the drop evaporates or you run out of crystals.
- Store frozen cryovials in a liquid nitrogen storage dewar for future use.

Notes

1. Each crystal should be labeled with a unique identifier so that it can be specifically identified later for diffraction screening and data collection. For example, Human carbonic anhydrase II crystals might be labeled HCAII-01, HCAII-02, etc. Cryocanes can be labeled with the first of a sequence of vial names contained within them for easy location in the storage dewar.
2. Drop evaporation will be especially problematic during the winter months, when indoor humidity levels are very low. Working at 4 °C may minimize this problem.

SOAKING-IN LIGANDS

Occasionally, it is desirable to determine a protein structure in the presence of a bound small molecule. One method of preparing such protein-ligand complexes is to soak a crystal in artificial mother liquor containing an excess of ligand; this can be done at the same time as cryoprotection if desirable and practical. Typically, the concentration of ligand used should be 10-1000x the dissociation constant (K_d) if it is known. Soaking for 10-30 min should be sufficient to populate the protein in the crystal if the binding site is accessible in the crystal lattice. If protein molecules pack in the crystal in such a way as to obscure the ligand-binding site, or if crystals do not tolerate extended soaking without cracking or dissolving, then "no-fail" cryoprotection or co-crystallization with ligand should be attempted.

"NO-FAIL" CRYOPROTECTION

This method is especially appropriate for crystals that cannot tolerate direct transfer to cryoprotectant solution, or for crystals that are especially sensitive to concentration changes in the mother liquor driven by drop evaporation. In our laboratory this method is routinely used with success on otherwise very sensitive crystals. This particular method is adapted for hanging drop crystallization. Ligands can be soaked in at the same time as cryopreservation if included in the cryoprotectant solution at 125% of the final, desired concentration. This method is typically gentle, but in our hands occasionally results in a slight but significant increase in crystal mosaicity. This increase in mosaicity can be reduced by shortening the soak time.

- Prepare a solution of artificial mother liquor + cryoprotectant[1] at 125% of the final, desired concentration.
- Remove a coverslip containing a drop with crystals to be cryoprotected and add 0.25 drop volume (DV) of cryoprotectant solutions (*e.g.* for a 4 µL drop add 1 µL of cryoprotectant solution). Replace coverslip on well and let stand for 0.5-5 minutes. Examine the crystals for cracking and/or dissolution.
- Repeat the previous step with the following additional cryoprotectant additions: 0.25 DV, 0.50 DV, 1.00 DV, 2.00 DV. After each addition replace the coverslip over the well and let stand for 0.5-5 minutes. Examine crystals for cracking and/or dissolution.
- After the last addition and 0.5-5 minute incubation, remove coverslip, fish out crystals with mounting loops and freeze directly in liquid nitrogen.

Notes

1. Glucose is a nearly universally tolerated cryoprotectant, and will dissolve in both PEG and salt solutions. The artificial mother liquor + cryoprotectant can be easily made by adding 375 mg glucose to a 1.5 mL microcentrifuge tube and making up to the 1.0 mL mark with well solution. This will be 37.5% glucose. Mix thoroughly to dissolve. (An ultrasonic bath will make quick work of dissolving the glucose, which tends to clump badly.) The final concentration of glucose in the drop after completing the protocol will be 30%, sufficient to cryoprotect any crystallization solution.