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Chapter 10.2. Cryocrystallography techniques and devices

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10.2.1. Introduction

The ability to collect X-ray data from macromolecular crystals at cryogenic temperatures has played a key role in the more widespread and effective use of crystallography as a tool for biological research. Radiation-damage rates are greatly reduced at low temperature, often by orders of magnitude, making it possible to work with crystals that would otherwise diffract too weakly or decay too quickly. In particular, this radiation protection is essential for the full use of increasingly powerful synchrotron-radiation sources, and the coupling of the two permits the investigation of ever larger macromolecular complexes and the collection of higher-resolution data from nearly all samples. Cryogenic data collection has also allowed efficient experimental phasing using multiple-wavelength methods, as well as preservation of unstable samples and trapping of transient enzyme intermediates, an area that will undoubtedly continue to gain in importance.

In this chapter, practical aspects of cryocrystallography are discussed, with an emphasis on techniques and devices for crystal preparation and handling. A number of prior reviews covering these and other aspects of macromolecular cryocrystallography are available (Hope, 1988, 1990; Watenpaugh, 1991; Rodgers, 1994, 1997; Abdel-Meguid *et al.*, 1996; Garman & Schneider, 1997; Parkin & Hope, 1998). Radiation-damage protection at low temperature, which is not well understood, has also been discussed (Henderson, 1990; Gonzalez & Nave, 1994; Rodgers, 1996; Garman & Schneider, 1997), and the principles and operation of cryostats for sustained cooling during data collection are described elsewhere (Rudman, 1976; Hope, 1990; Garman & Schneider, 1997).

10.2.2. Crystal preparation

Macromolecular crystals are intimately associated with bulk aqueous solution. It surrounds them and penetrates them as solvent-filled channels, which typically account for 30–80% of the crystal volume. A key goal, therefore, of any procedure for cooling these samples to cryogenic temperatures is to prevent the formation of hexagonal crystalline ice. Ice formation, because of the associated increase in specific volume, inevitably disrupts the order of the macromolecular crystals and renders them useless for data collection. The principle that underlies current methods is that sufficiently rapid cooling causes the formation of a rigid glass before ice nucleation can occur. The high viscosity of the glass then prevents subsequent rearrangement into an ordered lattice. Early attempts to flash cool macromolecular crystals were made by Low *et al.* (1966), Haas (1968), and Haas & Rossmann (1970).

With samples as large as macromolecular crystals, however, it is not possible to achieve the high cooling rates necessary to prevent ice formation in water and most aqueous crystallization solutions. The most general method for overcoming this problem is to equilibrate the crystal with a solution containing a cryoprotective agent that slows ice nucleation and allows the formation of a glassy solid with attainable cooling rates. A list of cryoprotectants used successfully with macromolecular crystals is shown in Table 10.2.2.1. Typically, these cryoprotectants are included in the established stabilization or harvest solution at concentrations that range from 6–50%. Glycerol is frequently chosen for initial trials and appears to be a widely applicable cryoprotectant for both salt and organic precipitants. Concentrations of glycerol necessary to prevent ice formation in a number of typical crystallization solutions have been tabulated (Garman & Mitchell, 1996). Other compounds such as ethylene glycol and small sugars fall into this same class. Crystallization precipitants such as 2-methyl-2,4-pentanediol (MPD) or polyethylene glycol (PEG) can often simply be increased in concentration to provide sufficient cryoprotection. Ethanol, methanol and MPD are useful in relatively low-salt conditions. The listed stereoisomer of butanediol is a particularly effective cryoprotectant and can be used where other components of the solution, such as high salt, may limit the amount of cryoprotectant that can be added. Limitations owing to salt in the crystallization mix can also be overcome by transferring the crystals to a solution containing an organic precipitant before or during the introduction of the cryoprotectant (Singh *et al.*, 1980; Ray *et al.*, 1991; Wierenga *et al.*, 1992). Combinations of cryoprotectants have been used where a single cryoprotectant alone did not permit successful flash cooling.

It is rare that a crystal can be transferred without damage directly to a solution containing full-strength cryosolvent. Usually, the cryoprotectant must be introduced slowly to reduce stress on the crystal lattice. Methods of introducing cryoprotectant-containing solutions are listed in Table 10.2.2.2. Techniques such as serial transfer through increasing cryoprotectant concentrations or dialysis are preferred. They allow the crystal to equilibrate with the cryosolvent, leading to reproducible crystal quality and unit-cell dimensions after flash cooling. Also, with equilibrium methods, the solution conditions can be altered in an

Table 10.2.2.1
List of cryoprotectants used successfully for flash cooling macromolecular crystals

See the text as well as Rodgers (1994, 1997), Abdel-Meguid *et al.* (1996), and Garman & Schneider (1997) for additional details.

(2*R*,3*R*)-(−)-Butane-2,3-diol	2-Methyl-2,4-pentanediol
Erythritol	Polyethylene glycol 400
Ethanol	Polyethylene glycol 1000–10 000
Ethylene glycol	Propylene glycol
Glucose	Sucrose
Glycerol	Xylitol
Methanol	

Table 10.2.2.2
Methods for introducing the cryoprotectants needed for flash cooling

(1) Serial transfer into increasing strengths of cryoprotectant
(2) Dialysis
(3) Growth in cryoprotectant
(4) Brief transfer before flash cooling
(5) Direct transfer into full-strength cryoprotectant

attempt to control any crystal damage associated with the flash-cooling process itself. The best scheme for serial transfer must be determined empirically. Equilibration time at each step depends on a number of factors (size of the crystals, solvent content, viscosity of the solution) but can be as rapid as less than a minute for small cryoprotectants or as long as hours for large polymers (Bishop & Richards, 1968; Fink & Petsko, 1981; Ray *et al.*, 1991). Typically, 5% increments in cryoprotectant concentration with equilibration times of 15 min or longer at each step are used in initial trials. The step size is then decreased if damage occurs. Dialysis can be done conveniently in the small buttons used for crystallization. These are available with chamber sizes as low as 5 µl and allow a piece of dialysis membrane to be stretched and held securely over the opening. Dialysis times range from 1 to 24 h depending on the size of the cryoprotectant and the viscosity of the solution.

Another technique, growth of the crystals directly in cryoprotectant solutions, is particularly convenient and effective. In some cases, the primary precipitant, MPD for example, may provide cryoprotection if the concentration used in crystallization is sufficiently high. More commonly, however, additives such as glycerol are included in the crystallization buffer. An advantage of this technique is that crystals can be mounted directly from the crystallization drop, eliminating potential damage in transferring to a harvest or cryoprotective solution.

When it is not possible to identify a cryosolvent compatible with the crystals, a brief exposure to the cryoprotective solution may allow successful flash cooling. Apparently, the water in crystal solvent channels is constrained sufficiently to prevent nucleation, and simply exchanging the external aqueous solution with cryosolvent provides protection. The 'quick dunk' in the cryosolvent may be as short as a few seconds, and for some crystals it is possible to combine this technique with prior equilibration in lower, non-damaging concentrations of cryoprotectant. The same principle of preventing ice formation in the external solution forms the basis of an alternative technique developed by Hope (1988). Here, the external solution is replaced by a hydrocarbon oil before flash cooling.

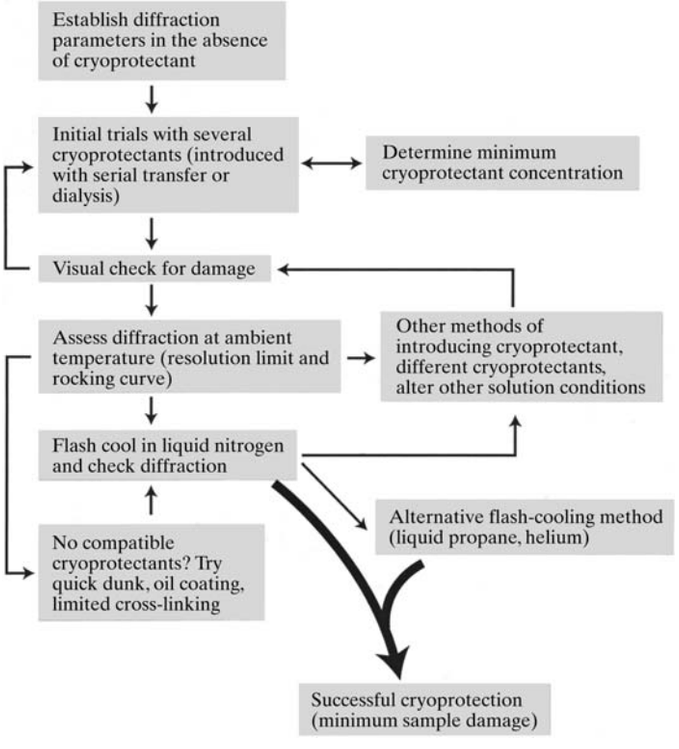

Figure 10.2.2.1
Recommended pathway for optimizing cryoprotectant conditions and flash cooling.

Finding suitable cryoprotection conditions is a trial-and-error process. Two problems must be overcome: the cryoprotectant must be introduced without significant damage to the crystal, and damage during the flash-cooling process must be minimized. A scheme for systematically determining conditions for flash cooling is given in Fig. 10.2.2.1. In order to assess the effect of subsequent manipulations, it is important first to establish the resolution and rocking curve of the crystals under normal harvest conditions. Then one or a few cryoprotectants can be added to the harvest solution under conditions that allow equilibration with the crystal. The minimum concentration of cryoprotectant necessary to prevent ice formation can be determined by flash cooling candidate solutions using the loop-mounting technique described in Section 10.2.3. A sufficient concentration of cryoprotectant will result in a transparent glass upon cooling, while too low a concentration will produce opaque microcrystalline ice. A solution of cryoprotectant 2–3% above this minimum value should be used to allow for the added volume and therefore slower cooling when the crystal is present. If the crystals crack or dissolve in a cryosolvent, then the cryoprotectant should be introduced more slowly, the solution conditions (precipitant concentration, ionic strength, pH) altered, or the cryoprotectant eliminated from consideration.

The diffraction quality of crystals that show no visible sign of damage should be assessed at the crystal-growth temperature, and solution conditions should be altered if there has been a significant loss in resolution or an increase in rocking-curve width. For crystals that are incompatible with a wide range of cryosolvent conditions, quick-dunk and oil-coating techniques should be considered. Limited cross-linking (with glutaraldehyde, for example) can sometimes stabilize crystals for the introduction of cryoprotectant or improve stability during flash cooling.

When conditions that result in little or no damage have been identified, the crystals should be flash cooled and the diffraction assessed again. The formation of even small amounts of microcrystalline ice can be detected after flash cooling as characteristic powder rings at low-order spacings of 3.90, 3.67, 3.44 Å. If ice forms, a greater concentration of cryoprotectant must be used. An increase in the rocking-curve width of the crystal at this stage is common, probably due to the thermal stress on the lattice or changes in solution properties on cooling. If this increase is more than 50%, or if any loss of resolution occurs, solution conditions should be altered and the process repeated. The concentration of the cryoprotectant can be increased and different cryoprotectants tested. Other solution parameters, as noted above, can also be adjusted in an attempt to decrease the damage from flash cooling. In addition, different flash-cooling techniques (discussed below) can be tested to determine whether they produce less damage. Suitable cryosolvent conditions are usually established after a few trials, and even in difficult cases it has generally proven possible to find acceptable conditions by continuing to refine solution parameters.

10.2.3. Crystal mounting

A mounting technique suitable for flash cooling should allow for rapid heat exchange by providing a large surface area and a minimum of extraneous material that must be cooled. The technique should also subject the crystals to little mechanical