Cryocrystallography

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Crystallographic studies are being extended to increasingly challenging systems: ever larger molecules, more complex viruses, membrane proteins, and assemblies. The crystals are often radiation-sensitive and scatter X-rays weakly, in many cases posing severe data collection problems. Synchrotron sources, high-performance area detectors and enhanced computational power have made it possible to tackle more difficult crystals and have greatly improved and simplified collection in general. Another tool, data collection at cryogenic temperatures, addresses more directly some of the problems associated with macromolecular crystals. Low-temperature techniques are coming into widespread use, and have become the routine method of data collection in a few laboratories. Cryogenic collection can make possible otherwise intractable projects, helping to expand the range of applications of crystallography to include some of the most exciting problems in biology. It is now clear that low-temperature techniques also offer real advantages with more conventional crystals, so that they should be considered for all macromolecular X-ray studies.

The primary benefit of data collection at cryogenic temperatures is a reduction in the rate of radiation damage, which often substantially extends crystal lifetime. Frequently, an entire data set can be collected from a single crystal when many would otherwise be required. But cryocrystallography offers other advantages that can contribute to the ultimate success of a data-collection effort. In many cases, the highest resolution to which accurate data can be recorded increases, either because of a reduction in thermal disorder or simply as a result of longer exposure times and a more stable crystal. The mounting techniques used are gentler and lead to less background scatter than conventional methods, and long-term storage and re-use of crystals are also possible. Finally, low-temperature techniques can be used to stabilize transient states, such as catalytic intermediates, that are too short-lived for study at higher temperatures. What are the disadvantages? Initially, of course, the complexity and cost associated with data collection increase, although with experience the techniques prove straightforward and the greater productivity more than offsets the additional expense. A more serious problem is that some crystals prove difficult to cool without unacceptable lattice distortion or increase in mosaic spread. In most cases, however, conditions can be defined which minimize the detrimental effects.

Flash-cooling

The principal challenge of cryogenic data collection is to cool the crystal without damaging it. At minimum, this requires the prevention of crystalline ice formation in the internal and external aqueous solution. Various strategies have been used (see [1–4] for descriptions), but the technique that has proven most effective and generally applicable is flash-cooling [5–7]. Here, the temperature of a sample is rapidly lowered (to ~100 K), so that ice nucleation does not occur before the viscosity of the solution is high enough (below the glass transition temperature) to prevent ice lattice formation (see [8,9]). The result is a rigid glass that encases the crystal lattice. Even with the fastest cooling rates, however, ice will form in most harvest solutions and severely damage the crystal. One approach is to remove nearly all the external liquid from the crystal by transferring it to a hydrocarbon oil [1,10]. In a number of cases (see [3]), samples can then be flash-cooled satisfactorily. Many crystals (particularly the more challenging ones) are damaged with this technique, either by handling, incompatibility with the oil, or subsequent flash-cooling. A more general method is to prevent ice formation during flash-cooling by adding cryoprotectants [5,10,11]. Combined with the crystal-mounting technique described below, this approach has proven successful for a broad range of samples, and it is now the most widely used method for cooling to cryogenic temperatures (see [12] for a detailed description).

Cryoprotectants

Added cryoprotectants are not intended to maintain the harvest solution in a liquid state. They simply slow nucleation so that a rigid glass is formed before ice formation occurs. (Added solute also affects the viscosity of the solution as it cools, raising the glass transition temperature (see [9]), so that less time is available for ice nucleation during flash-cooling.) The effectiveness of compounds as cryoprotectants, or more precisely as glass-forming agents, varies considerably [13]. Even closely related compounds can have widely different cryoprotective abilities. For example, (2*R*,3*R*)-(–)butane-2,3-diol is a particularly effective cryoprotectant, but mixtures containing the *meso* isomer can be much poorer glass-forming agents [13]. Fortunately, fairly high cooling rates (see below) can be attained with samples the size of macromolecular crystals. This allows one to consider a number of compounds when searching for one compatible with the crystals at a concentration that insures glass formation. A list of cryoprotectants that have been used successfully is given in Table 1. Glycerol is often tried first, and it was used most frequently among the studies surveyed in producing Table 1. Ethylene glycol, low-molecular-mass polyethylene glycol, and 2-methyl-2,4-pentanediol (MPD) are also popular. For the most part, the remainder have been used when these cryoprotectants did not give satisfactory results.

Table 1. List of cryoprotectants used successfully in flash-cooling macromolecular crystals.

Type	Concentration (%)
Glycerol	13–30 (v/v)
Ethylene glycol	11–30 (v/v)
Polyethylene glycol 400	25–35 (v/v)
Xylitol	22 (w/v)
(2*R*,3*R*)-(−)-butane-2,3-diol	8 (v/v)
Erythritol	11 (w/v)
Glucose	25 (w/v)
2-methyl-2,4-pentanediol (MPD)	20–30 (v/v)

The list was compiled from unpublished observations in the laboratories of Stephen Harrison and Don Wiley at Harvard University and a survey of structures and reports in six journals for the years 1993 and 1994. Forty different crystals are represented. The range of reported concentrations for each cryoprotectant is also given.

Obtaining good cryoprotectant conditions is straightforward in most cases. The simplest approach is to begin by including candidate cryoprotectants in the harvest solution. The minimum concentration needed will depend on the additive and the other components of the harvest mix. To avoid damaging the crystal, it is usually necessary to introduce the cryoprotectant slowly, either by dialysis or by serial transfer, and if possible, the crystal should be allowed to equilibrate fully before flash-cooling. Permitting full equilibration generally eliminates problems associated with non-isomorphism. In a number of cases, however, exposing the crystal to the cryoprotectant for a brief period has given good results. This method is particularly useful when the crystal is not stable for long periods in cryoprotectant solutions. Other methods of achieving satisfactory cryoprotection have been reported. It is possible, for example, to transfer some crystals from the normal mother liquor to unrelated solutions that contain cryoprotectant [14–16]. Another convenient approach is to grow the crystals in the presence of cryoprotectant, eliminating the need for a potentially damaging transfer to harvest buffer.

Even when ice formation is prevented, some crystals will still be damaged by flash-cooling. This can be the most troublesome aspect of the technique. The damage is evident as a broadening of the rocking curve, sometimes coupled with a significant decrease in the diffraction limit. It may result from stress induced by thermal contraction (possibly differential contraction of the crystal and solvent) or changes in solution parameters (for example dielectric) as the sample cools. If resolution is not lost, relative to crystals at ambient temperature, the increase in rocking-curve width can often be tolerated. An effort should be made to minimize any significant damage, however, by varying solution parameters. Changing the type or increasing the concentration of the cryoprotectant can be helpful. Alterations in other solution parameters such as ionic strength, pH and buffer system should also be considered, either for their initial effect on the strength of the lattice, or to compensate for changes during cooling. Usually, some combination of parameters that eliminates the damage or reduces it to acceptable levels can be found.

Mounting, cooling and storage

Cryocrystallography is becoming more widely used in part because simple, effective methods for cooling and maintaining the crystals at low temperature are now available. A major advance was the introduction by Teng [17] of a novel crystal-mounting technique intended primarily for flash-cooling. The crystal is held in a thin film of cryoprotectant-containing solution formed within a small loop (Fig. 1). The method places little mechanical stress on the crystals, so it is excellent for fragile samples. (In fact, it can be used for crystals at ambient temperatures by sealing the loop and a reservoir of harvest solution within a large-diameter X-ray capillary.) This technique also exposes a large surface area, which is important for achieving high cooling rates. Originally, thin metal wire was used to form the loop, but the X-ray opaque wire creates blind regions where data can not be measured. Loops are now made from very fine (~10 μm diameter) fibers of nylon or glass wool [12]. Error from absorption by these loops is small, particularly if excess solution is minimized by making the loop just large enough to accommodate the crystal (see Fig. 1). With this mounting technique, background scatter and absorption are much reduced compared with crystals placed in standard glass capillary tubes [17].

Two methods are commonly used to rapidly cool the crystals once they are mounted in a loop. Placing the crystal directly in the stream of cold nitrogen gas from a cryostat is probably the most common flash-cooling technique (see [2,12]). (The principles of cryostat operation have been described [2,18], and several excellent commercial cryostats are now available.) The procedure is simple, and because it is usually performed on an X-ray camera, the crystal is already in position for analysis. However, it probably does not afford maximum cooling rates (H Hope, LJ Walker, PO Moreno, abstract PE01, American Crystallographic Association Meeting, Atlanta, 1994), requires the availability of a cryostat, and is not efficient when cooling and storing a large number of crystals (see below). Alternatively, the crystal and loop can be plunged directly into a cryogenic liquid [5–7]. Higher cooling rates can be achieved using this technique, and a cryostat need not be available when preparing samples. Using standard cryogens (ethane, propane, and some types of freon) is, however, more difficult and potentially more dangerous (because of flammability) than flash-cooling in a gas stream. Liquid nitrogen, although employed successfully in an early study [5], has been considered unsuitable for this technique because it was thought to form an insulating layer of gas around the sample, slowing heat transfer [9]. But recent measurements suggest that the cooling rates with liquid nitrogen and standard cryogens are more comparable than expected (H Hope, LJ Walker, PO Moreno, abstract

Fig. 1. Photograph of a flash-cooled crystal mounted in a fiber loop. The crystal was picked up with the loop (left side of the figure) from a drop of harvest buffer and flash-cooled in the nitrogen gas stream from a commercial cryostat. The loop was made by forming it around a wire support and twisting the free ends to form a long stem, which was then coated with glue to both stiffen it and prevent unraveling. The stem was cemented to a wire support (visible on the right-hand side of the figure), which was attached to a steel base (not shown). The loop diameter is ~0.25 mm.

PE01, American Crystallographic Association Meeting, Atlanta, 1994). Although additional information on cooling rates is needed, we have had success flash-cooling directly into liquid nitrogen. Avoiding the other cryogens greatly simplifies the technique, and many samples can be prepared in a short time.

Both methods of flash-cooling require a rapid, trouble-free mechanism for transferring the sample to the X-ray camera. When cooling in the gas stream, evaporation from the loop-mounted sample is rapid because of the large surface area, and it must be handled very quickly (often within only a few seconds) to prevent drying. When flash-cooling directly in liquid cryogen, the sample must be transferred to the cryostat gas stream without warming. A magnetic system has been developed [12] to permit rapid attachment of the loop to a goniometer on the X-ray camera. A magnetic strip is attached to the top of a goniometer, and the loop assembly, which has a steel base, can then be placed on and off quickly. A locating pin on the goniometer guarantees that the loop is positioned accurately in the cold stream of the cryostat. This magnetic holder is also the basis of a system for storing crystals in liquid nitrogen using standard cryogenic dewars. Storage of the crystals allows flash-cooling to be done in any convenient location, such as a cold room for example. The crystals can then be transferred to the X-ray camera when needed. This system is particularly useful when preparing samples for data collection at synchrotron radiation sources, allowing more productive use of the limited time available at these facilities. Crystals can be prepared under standardized conditions, prescreened and catalogued in the laboratory, and transported in liquid nitrogen to the synchrotron source. Some crystals degrade in growth and harvest solutions, and liquid nitrogen storage is an excellent way of stabilizing crystals for long periods. It also allows crystals to be re-used if, for example, additional data are required.

Comments and examples

Many of the results and experiences with flash-cooling have been presented in earlier reviews [2,3], and a comprehensive treatment will not be attempted here. The recent structure determination [19,20] of the low-pH form of soluble influenza virus hemagglutinin fragment (TBHA2) illustrates the usefulness of flash-cooling techniques. Crystals were grown from 55%–65% ammonium sulfate in space group $C222_1$, with unit cell dimensions of a=174.7 Å, b=231.8 Å and c=54.5 Å, and with one trimer of 57 kDa molecular mass per asymmetric unit (70% solvent content). At room temperature, they were highly radiation-sensitive and only a small portion of a data set could be obtained from a single crystal. Accurate data could be collected to only ~4.0 Å using a rotating anode X-ray source, and it was difficult to construct a complete set by merging data from a number of crystals. In addition, the protein supply was limited, and derivatives were required. Fortunately, it was easy to find good cryoprotectant conditions. Crystals were transferred through a series of harvest solutions containing increasing concentrations of glycerol (5% to 20% (v/v) by 5% steps at room temperature), with at least 30 min equilibration at each step. They were then flash-cooled in the nitrogen gas stream at ~110K. The full width of the rocking curve increased only slightly, from ~0.4° at room temperature to ~0.5° at low temperature, and the cell constants decreased to a=168.7 Å, b=231.7 Å and c=53.8 Å. With flash-cooled TBHA2, a complete data set could be collected from a single crystal without radiation decay. The quality of these data were somewhat better than partial data sets from room temperature crystals (Fig. 2a). Subsequently, higher-resolution native data were collected at a synchrotron source from one crystal, and the structure was determined using two derivatives collected (using a rotating anode) from single crystals at low temperature.

An increase in mosaic spread during flash-cooling was not a serious problem with TBHA2 (although the rocking-curve width at room temperature was already high). Recent experience with rocking-curve changes is not in general well documented, but at Harvard University a survey of 19 different crystal systems found eight crystals with little or no increase in rocking-curve width, three crystals with an increase of 50% or less, and eight crystals with an increase of around 100% (for example 0.2° increasing to 0.4°). These results appear to be consistent with those from other laboratories using similar techniques. Although a broadening of the rocking curve must have some adverse effect on data quality, it is usually more than counterbalanced by the positive effects of cryogenic collection. Current detectors and software help to minimize the effects of a broader rocking curve. Every effort should be made to reduce or eliminate any increase, but even when broadening of the rocking curve is still substantial, cryogenic collection can often be the best route to completion of the project.

The TBHA2 unit cell decreased in volume by ~4.7% on flash cooling. This volume change is typical [4,21–24], although values can vary and larger changes occasionally occur (possibly when cryoprotectant is introduced). The differences between room-temperature and cryogenic structures of the same proteins have been analyzed in a number of cases [21–26]. Thermal expansion is not uniform, but results from movements of secondary and tertiary elements, with predominantly α-helical regions having slightly higher expansion coefficients than mainly β-sheet domains. However, these effects only cause small differences in the relative positions of secondary structure elements (up to a few tenths of an Ångstrom), and they are not a concern in most studies. Cooling to cryogenic temperatures can reduce refined atomic B-factor values, particularly for more ordered crystals. Side chains may become better ordered, and occasionally they adopt significantly different conformations. Also, a larger number of ordered solvent molecules are usually found at cryogenic temperatures. Where lower atomic B-factor values have been reported, the resolution limit of diffraction may increase [2,4,27]. Even when this effect is not significant (i.e. for crystals with greater static disorder), the advantages of data collection at cryogenic temperatures (single crystal, longer exposure times, low background and absorption) usually result in significantly improved data statistics and an effective increase in resolution.

Previous reports have emphasized the apparent lack of radiation damage with flash-cooled crystals using rotating anode X-ray generators [2,3] and synchrotron sources [27]. Although the improvements in stability under these conditions are dramatic, radiation damage does still occur and has recently been characterized [28]. As in room-temperature data collection, the effects of the damage are a decrease in the intensity of reflections, progressing from high to low resolution, and a broadening of the rocking-curve width (Fig. 2b). The useful lifetime of a crystal is roughly 5×10^{15} photons mm^{-2}, close to the estimate of

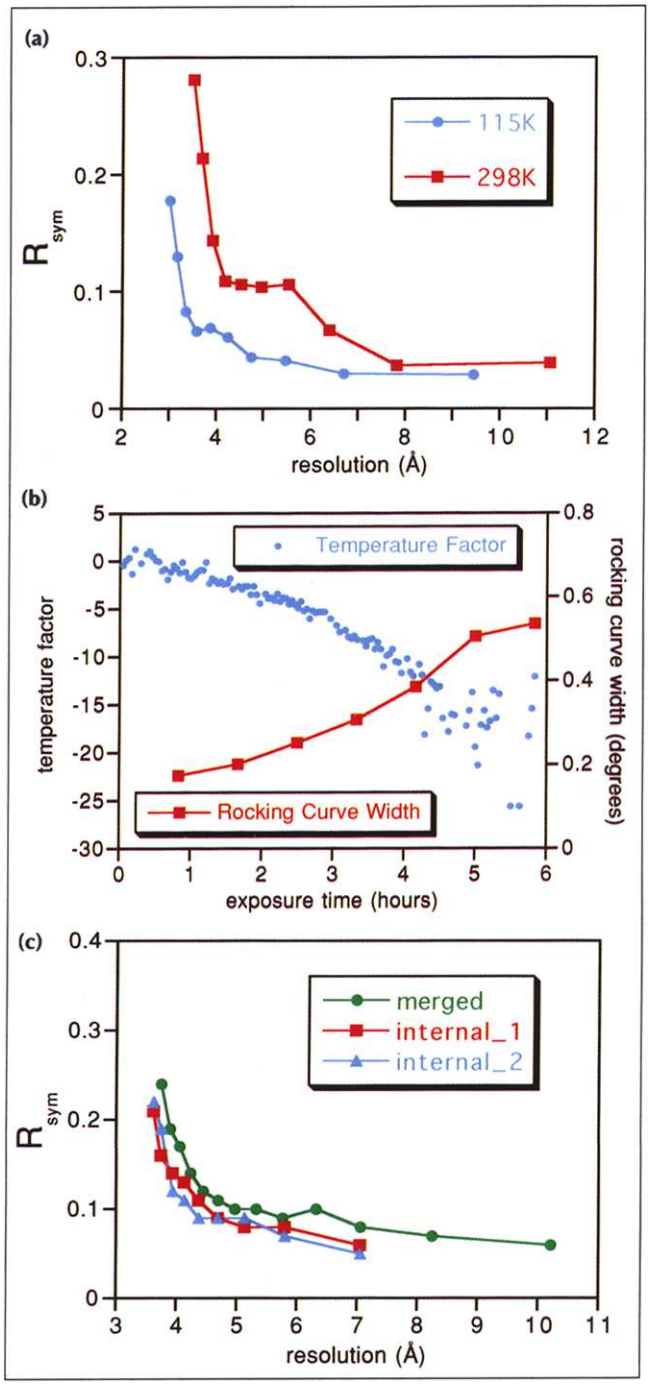

Fig. 2. Results with flash-cooled crystals. **(a)** Plot of merging statistics for single TBHA2 crystals [19,20]. $R_{sym}=\Sigma_{ij}|I_{ij}-\langle I_i\rangle|/\Sigma_{ij}|I_{ij}|$, where I_{ij} is the intensity of an individual measurement and $\langle I_i\rangle$ is the mean value for all measurements of each independent reflection. The data collected at 298 K are a partial set and the data from the crystal at 115 K are a complete set. **(b)** Crystal decay at cryogenic temperature. The scaling temperature factors for successive data frames and refined rocking curve width are plotted against exposure time. The data were collected from one crystal of influenza virus hemagglutinin (HA) [28]. The scaling temperature factor is given by $I_{scaled}=k_i I_{obs}\exp(-B_i\sin^2\Theta/\lambda^2)$ where k_i and B_i are the scale factor and temperature factor respectively for the ith batch. The rocking curve estimate is for full peak width. **(c)** Merging of data from a number of flash-cooled crystals. Statistics are plotted for two single crystals and a combined data set from a number of crystals of the reverse transcriptase from HIV-1 [30].

Henderson [29] based on experience with electron irradiation. As proposed by Henderson, the rigid glass encasing the crystal at cryogenic temperatures may prevent movement of radiolytic fragments and damage to one molecule from propagating to nearby portions of the lattice. This mechanical support would then be the mechanism responsible for the increase in crystal lifetime. The decay that does occur would result simply from the damage to individual molecules, and it should therefore be relatively independent of crystal type. Indeed, different crystals do show similar rates of decay at cryogenic temperatures. For the mosaic spread to increase, however, larger distortions of the crystal lattice must still occur to some extent. At the observed rate of decay, crystals are effectively free of damage when irradiated by rotating-anode sources, and lifetime considerations may be ignored when setting exposure times. At high-intensity synchrotron sources, however, trade-off between crystal decay and exposure time is still required for weakly scattering crystals. In most cases, as with TBHA2, selection of exposure times so that a complete data set can be collected from one crystal will give an acceptable resolution limit. When the data quality achieved using this approach is not adequate, longer exposure times will be necessary and multiple crystals must be used to construct the data set. The structure determination of the unliganded form of reverse transcriptase from the human immunodeficiency virus is an extreme example of the need for multiple crystals [30]. Small crystals (0.15 mm×0.40 mm ×0.02 mm) grow in space group C2 with a large unit cell (a=168.7 Å, b=162.8 Å, c=331.8 Å) and four molecules of 117 kDa molecular mass each per asymmetric unit. With the small crystal size and large cell dimensions, long exposure times at a synchrotron source are required to record any diffraction to higher resolution than 3.5 Å. Only ~5° of data could be collected from a single crystal at low temperature, and 34 crystals were used to construct the final data set. As shown in Fig. 2c, the merging statistics for data from individual crystals agree well with data combined from a number of crystals, indicating that they are acceptably isomorphous.

Outlook

Routine cryogenic data collection from macromolecular crystals is now possible, and the technique will continue to gain in popularity as more difficult problems are attempted. Recently, for example, two types of virus crystals have been successfully flash-cooled: rotavirus (BRS Temple, BA Harris and SC Harrison, personal communication) and cowpea mosaic virus/rhinovirus 14 chimera (C Momany, T Lin and JE Johnson, personal communication). In addition, the combination of cryogenic methods and phasing by multi-wavelength anomalous diffraction (MAD) is particularly powerful, simplifying data collection and extending the MAD technique to radiation-sensitive crystals. A number of structures have now been determined in this way: human chorionic gonadotropin [31], lacR core fragment (AM Friedman, TO Fischmann and TA Steitz, personal communication), phage T4 gene32–ssDNA complex (Y Shamoo, AM Friedman, MR Parsons, WH Koenigsburg and TA Steitz, personal communication) and NFκB p50 homodimer (C Mueller, *et al.*, personal communication). Finally, one of the most exciting applications of flash-cooling is the study of transient or intermediate states in enzymology, and several examples have already been reported (X Ding, BF Rasmussen, GA Petsko and D Ringe, abstract E02, American Crystallographic Association Meeting, Atlanta, 1994) [26,32]. It is clear that, in many ways, the flash-cooling method is becoming an essential tool for the macromolecular crystallographer.

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