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A General Method for Hyperquenching Protein Crystals

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Abstract

During flash cooling of protein crystals in liquid cryogens, cooling rates are determined by sample size, choice of cooling liquid, and by the thickness of the cold gas layer that forms above the liquid. We describe an experimental protocol for ultra-rapid cooling of protein crystals. This protocol requires no complex apparatus, and yields ice-ring-free diffraction without the use of penetrating cryoprotectants.

Keywords

cryocrystallography; cryoprotectant; flash cooling; hyperquenching; protein crystallography; vitrification

Introduction

Cooling of protein crystals to $T=100$ K dramatically reduces radiation damage, and allows complete data sets to be collected from single crystals (Hope 1988, Rodgers 1994, Chayen *et al.* 1994, Garman & Schneider 1997). However, cooling damages crystals, broadening their mosaicity and often decreasing the achievable resolution. Diffraction rings from crystalline ice often appear, and these interfere with diffraction from the protein lattice (Garman 1999, Juers & Matthews 2001, Juers & Matthews 2004).

Naively, one can imagine two limiting strategies for cooling a protein crystal to $T=100$ K. The first is to cool so slowly that the sample remains in quasi-equilibrium (Drew *et al.* 1982). In the absence of solvent vitrification/crystallization, abrupt structural transitions and kinetic trapping, the internal solvent and solute concentrations, protein conformation and crystal packing may then adjust in a way that produces little osmotic shock or lattice strain and that yields a well-ordered low-temperature state. Large cryoprotectant concentrations and cooling times of hours may be required, and the resulting low-temperature protein conformation may differ in significant ways from the biologically relevant structure.

The other limit is to cool the crystal so rapidly that the solvent and solutes have little time to redistribute, the solvent does not have time to crystallize, and the protein conformation and packing have little time to relax, so that the room temperature structure and order is largely preserved (Halle 2004). No cryoprotectant is then necessary to prevent crystalline ice formation. When approaching this limit, heat transport through the crystal itself (rather than through the cooling fluid) becomes the bottleneck. Small crystals must then be used to obtain high cooling rates, and also to minimize internal thermal gradients and associated lattice stresses that may damage the crystal. Even when uniformly cooled, internal stresses associated with differential contraction of crystal constituents and with the non-equilibrium internal state may still cause cracking, especially for asymmetrically shaped crystals. Achievable cooling

times to $T=100$ K may be comparable to that achieved for vitrification of pure water, on the order of 1 ms (Bruggeller & Mayer 1980, Mayer 1985, Johari *et al.* 1987).

In current cryocrystallography practice, cooling times to $T=100$ K are of the order of 1 s (corresponding to cooling rates of 300 K/s to 1000 K/s), in neither the slow nor fast cooling limit. Here we describe sample preparation and cooling methods that dramatically reduce cooling times and allow protein crystals to be successfully cooled without the use of penetrating cryoprotectants. A detailed discussion of how these methods affect diffraction outcomes will be presented elsewhere.

Why do conventional flash cooling methods produce such slow cooling?

Protein crystals are typically cooled by inserting them into a cold nitrogen gas stream at $T=100$ K, by plunging into liquid nitrogen at $T_b=77$ K, or by plunging into liquid propane at $T\sim T_m=84$ K. Use of these different cooling media has not produced appreciably different diffraction outcomes. Two measurements of cooling rates using thermocouples of size comparable to protein crystals found cooling rates of 250, 500 and 1200 K/s and 70, 230 and 115 K/s for nitrogen gas, liquid nitrogen and liquid propane, respectively (Teng & Moffat 1998, Walker *et al.* 1998). However, analysis of heat transfer from protein crystals (Kriminski *et al.* 2003) and previous experiments in cryoelectron microscopy (for a review see Bald 1992) suggest that liquid nitrogen should provide at least an order of magnitude faster cooling than cold nitrogen gas, and that liquid propane should provide even faster cooling, especially of larger samples where cooling rates in liquid nitrogen are limited by nucleate boiling. Why are cooling rates in current protein cryocrystallography practice so slow, and why do they show so little variation with cooling medium?

A cold liquid will cool the gas immediately above it, and may evolve gas by vaporization. The thickness of the resulting cold gas layer adjacent to the liquid surface can be characterized using the height at which the temperature rises above water's glass transition temperature T_g (or above its homogeneous nucleation temperature T_h).

To understand the effect of this cold gas layer, let us again consider two limiting cases. First, imagine plunging a large sample such as your fist through the gas and into the liquid cryogen. Since the thermal mass of your fist is so large, its temperature changes little as it passes through the cold gas layer, and most cooling occurs once it enters the liquid. The cooling rate is then determined by the rate of heat transfer to the liquid. Now imagine plunging a very small sample — an atom, say — through the gas and into the liquid. Since the thermal mass in this case is so small, the atom will remain in quasi-equilibrium with its environment as it plunges. Its temperature will drop as it traverses the cold gas layer, reaching the temperature of the liquid just above the liquid's surface. The cooling rate will then be determined by the time to traverse the cold gas layer, and be independent of the heat transfer properties of the liquid.

The key question in determining the cooling behavior is then: What is the characteristic sample size above which cooling occurs primarily in the liquid, and below which cooling occurs primarily in the cold gas layer above it?

For plunge cooling in liquid nitrogen at the plunge speeds of ~ 0.5 m/s typical in current protein cryocrystallography practice, the answer — obtained from studies of vitrification of water-cryoprotectant mixtures — is ~ 100 nl (Berejnov *et al.* 2006, Warkentin *et al.* 2006). This volume corresponds to a crystal roughly 500 μm on a side, larger than all but the very largest protein crystals. Consequently, in current practice most of the cooling occurs in the gas layer! This explains the small differences in cooling rates and diffraction outcomes seen when crystals are cooled by insertion in gas streams or by plunging in liquid cryogens.

Cold gas layer removal and hyperquenching

How can we minimize cooling in the gas layer and maximize cooling rates? One way is to plunge the sample at high speed, so that the time during which it can be cooled by contact with the gas is very small. This approach is used in cryoelectron microscopy, but is impractical for the much bulkier samples and more fragile sample mounts used in protein crystallography. Another way is to simply remove the cold gas layer, so that the sample's environment abruptly transitions from room temperature to the liquid's temperature when it enters the liquid.

How can we remove the gas layer? By simply blowing across the surface of the liquid with warm (room temperature) dry nitrogen gas. Even very modest gas flow speeds of a few m/s can reduce the thickness of the cold gas layer from ~1 cm to less than 100 μm . With such thin cold gas layers, even relatively leisurely plunge speeds can eliminate their effect, and yield cooling rates comparable to or larger than can be achieved with the very high plunge speeds used in cryoelectron microscopy. For a 20 μm thermocouple plunged at 0.4 m/s into liquid nitrogen, removing the cold gas layer increases the cooling rate from 1800 K/s to ~20,000 K/s, and reduces the time to cool from T_h to T_g from 150 ms to ~13 ms. This represents a nearly two order-of-magnitude improvement over current best practice in protein crystallography (Warkentin *et al.* 2006).

Faster cooling reduces the cryoprotectant concentrations needed to prevent crystalline ice formation and obtain a vitreous low-temperature phase. For protein and salt-free mixtures of water and glycerol plunged into liquid nitrogen, removing the cold gas layer reduces the required glycerol concentration for ~50 μm (0.1 nL) samples from 28% to 10%. Protein at the extremely high concentrations found inside protein crystals is a very effective cryoprotectant. With faster cooling, little or no penetrating cryoprotectant should be necessary to prevent internal ice crystallization in most cases, allowing cryoprotectants to be eliminated from crystallization screens.

Sample preparation for hyperquenching

For given a given liquid cryogen and plunging conditions, the crystal's cooling rate can be increased by minimizing the sample's total thermal mass and maximizing its surface-to-volume ratio. This suggests the use of small crystals — much smaller than ~500 μm — surrounded by as little mother liquor or cryoprotectant as possible. Modern synchrotron beam lines allow protein structure determination with crystals as small as 5 μm (Coulibaly *et al.* 2007), and structure solution with crystals in the 20 μm to 50 μm range will soon be routine. Such small crystals will dehydrate very quickly — in a matter of seconds — if exposed to dry room temperature air.

We have found that low-molecular-weight perfluoropolyether (PFPE) oils (e.g., Fomblin oil) can be used to remove surrounding mother liquor, thinly coat the crystal, and provide excellent protection against dehydration. A drop of oil is placed next to (or on top of) the crystal-containing drop. The crystal and liquid adhered to it are transferred to the oil, and then to a large pool of oil on a separate coverglass to allow the crystallization well to be resealed. The crystal is now carefully moved back and forth in the oil until all the surrounding mother liquor has been removed, at which point the crystal will no longer leave visible trails of emulsified solvent, and its facets will almost disappear due to the close refractive index match between oil and crystal. By completely removing the surrounding mother liquor in this way, cryoprotectants like glycerol need not be added to prevent its crystallization on cooling. Finally, the layer of oil is carefully thinned by wicking or by tapping on the coverglass. Because of its low viscosity, the oil thickness can be reduced to a few micrometers, minimizing its contribution to the sample's thermal mass. Oil may provide an additional benefit of hastening the onset of nucleate boiling in liquid nitrogen and thus increasing cooling rates (Gakhar &

Weincek 2005). Figure 1 shows an example of a protein crystal mounted and cooled in Fomblin oil using this procedure.

Accidental annealing

While small crystals will cool very quickly, they will also warm very quickly if the temperature of their environment fluctuates during handling. Accidental annealing in this way is likely to undo the benefits of initial fast cooling. We have found that large temperature transients at the sample's position can occur when the sample is transferred from liquid nitrogen to the cold stream. The motions of the sample goniometer base, cryovial and/or gripper/tongs temporarily disrupt the flow patterns of both the inner cold gas stream and the outer warm gas stream. We have replaced the sample in a goniometer base with an 80 μm bead thermocouple, and measured the temperature as a function of time when an initially liquid-nitrogen filled magnetic cryovial is removed from the goniometer base in a standard cryostream. We observe significant transient warming: the temperature typically rises to ~ 150 K and in the worst case to ~ 240 K. Schlieren imaging shows that as the vial is removed, the outer warm gas flow sweeps across the sample, producing this transient warming. We have implemented a liquid nitrogen drizzle similar to that shown in Pflugrath 2004 (although we use it for a different purpose) that sprays the sample as soon as the vial separates from the base, as shown in Figure 2.) Using this drizzle together with vials that are initially filled with liquid nitrogen, the sample temperature always remains at or below $T=100$ K. Thus, inadvertent annealing is eliminated and the full benefits of ultra-fast crystal cooling can be realized.

Discussion and Conclusion

The combination of methods described above minimizes crystal dehydration, maximizes cooling rates, eliminates surrounding mother liquor and associated ice ring diffraction, and eliminates inadvertent annealing. Using these methods, we have obtained high quality diffraction with no ice rings from more than 100 crystals of 12 different proteins having solvent contents ranging from 40 to 56 % — without the use of any penetrating cryoprotectants. A quantitative comparison between the hyperquenching method and conventional methods has shown that faster cooling produces lower mosaicity (unpublished) both in model proteins and in recent structural genomics targets, and this work is ongoing. As these methods are adopted, the macromolecular crystallography community will provide additional evaluation of the efficacy of hyperquenching in improving diffraction outcomes. While these methods may be more complex than those used in current practice, they can be performed flawlessly and reproducibly every time. They should reduce or eliminate the need for penetrating cryoprotectants, and improve reproducibility of cryopreservation outcomes for all proteins.

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Abbreviations

PFPE, perfluoropolyether.

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Figure 1.

A Lysozyme crystal mounted and cooled using the methods described in the text. The crystal was transferred to a drop of Fomblin oil to remove surrounding mother liquor and retrieved using a MicroMount (Mitegen, Ithaca, NY) with a 75 μm diameter aperture. The oil was then carefully removed using a wick, leaving a very thin film, and the crystal plunged into liquid nitrogen. The circle in the crosshairs has a diameter of 100 μm , and the crystal thickness is less than 40 μm .

Figure 2.

A liquid nitrogen drizzle, used to eliminate temperature spikes at the sample position caused by cryoflow disturbance when the cryovial is removed. The image was taken immediately after mounting the goniometer base on the goniometer head and removal of the cryovial; the base is still cold and so the liquid nitrogen stream clings to it rather than boiling upon contact. A fine-wire thermocouple at the tip of the goniometer base replaces a crystal mount, and is used to record the temperature jump from 77 K (in liquid nitrogen) to 100 K (in the cryostream) during mounting from a storage dewar. The liquid nitrogen drizzle eliminates the temperature spike above 100 K observed without the drizzle.