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PART 10. CRYOCRYSTALLOGRAPHY

Chapter 10.1. Introduction to cryocrystallography

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Since about 1985, low-temperature methods in biocrystallography have evolved from stumbling experimentation to mainstay production techniques. This would not have happened without good reason. Most of the early development was done with nitrogen cooling. More recently, temperatures as low as 5 K have become accessible by means of liquid-helium-based techniques. A brief discussion of the methods, equipment and advantages of data collection at cryogenic temperatures is given.

10.1.1. Cooling of biocrystals

10.1.1.1. Physical chemistry of biocrystals

Crystals are normally brought from room temperature to the working, low temperature by relatively rapid cooling, either in a cold gas stream, or by immersion in a cryogen such as liquid nitrogen or liquid propane. One goal of the procedure is to avoid crystallization of any water present in the system, whether internal or external to the crystal. Ice formation depends on the formation of nuclei. Nuclei are formed either by homogenous nucleation, *i.e.* in bulk liquid, or by heterogeneous nucleation, *i.e.* at the surface of a phase other than the liquid. Although data pertaining to biocrystals are scarce, indications are that internal nucleation, whether homogenous or heterogeneous, is not common. Proteins that induce nucleation at mild supercooling are known, so presumably there exist regions in these proteins that help to prearrange water molecules so that they readily form ice nuclei. There are also proteins that hinder nucleation. At present there is no basis for predicting the outcome of cooling for any given protein crystal. Only in a statistical sense can one be reasonably confident that a given macromolecule will not promote the freezing of water.

Vali and coworkers (Götz *et al.*, 1991; Vali, 1995) have provided a quantitative treatment of ice nucleation that can serve as a guideline. They observe that the absolute rate of formation of nuclei increases with the volume of water and with decreasing temperature. The probability p that a volume V of water will begin freezing during a time span t is given by

$$p = J(T)Vt,$$

where $J(T)$ is the nucleation rate at temperature T. Based on empirical data, $J(T)$ is given by

$$J(T) = 6.8 \times 10^{-50} \exp[3.9(273 - T)],$$

where J is in $m^{-3}\ s^{-1}$ and T is in K. Note that $J(T)$ increases by a factor of 50 per K. As a practical limit, bulk water cannot be cooled below 233 K without freezing. However, given a sufficiently small volume and high cooling rate, it is possible to supercool water to form a glassy state that is at least kinetically stable. Stability requires a temperature below 140 K; at higher temperatures crystallization eventually takes place. For the cooling rates typically attained with small crystals (up to a few hundred $K\ s^{-1}$) it seems impossible to avoid crystallization of water in the mother liquor adhering to a crystal, unless it is modified in some way. Once ice forms at the crystal surface, freezing may propagate through the entire crystal, effectively destroying it. Even if the crystal remains intact, diffraction from polycrystalline ice will render parts of any data set from that crystal useless. Because the probability of a nucleation event increases with time, it seems prudent to use a rapid cooling process. However, we note that the expression for $J(T)$ is formulated for pure water and cannot be valid for all conditions; it is well established that a majority of biocrystals can be cooled below 140 K.

A consequence of the foregoing is that for prevention of ice growth one should first focus attention on the region immediately outside the crystal, rather than on its interior. Two approaches have been shown to have merit: (*a*) modification of the solvent layer, and (*b*) removal of the solvent layer.

The goal of solvent modification is to prevent ice formation in that layer. Commonly used modifiers (referred to as antifreezes or cryoprotectants) are water-soluble organic compounds of low molecular weight with good hydrogen-bonding properties; examples include glycerol, ethylene glycols and MPD (2-methylpentane-2,4-diol). These compounds are added to reach a concentration sufficient to suppress nucleation and thereby prevent ice formation. Typical concentrations are in the 15–30% range, depending on the compound and the original composition of the mother liquor. The required concentration must be determined by experiment. Some suitable starting points are given by Garman & Mitchell (1996). The modified solution is tested by cooling a small drop to the working temperature. If the drop remains clear, there is no ice formation.

It is important to keep in mind that any change in the properties of the medium surrounding the crystal will have consequences for its crystallographic stability. In order to protect the crystal, two fields should be considered: thermodynamics and kinetics.

For a crystal in equilibrium with its mother liquor, the chemical potential of each species will be the same inside the crystal and in the mother liquor. If the solution surrounding the crystal is altered by the addition of an antifreeze, the chemical potential μ of water (and other species) will change and the crystal will no longer be in equilibrium with its surrounding solution. The typical result is that $\mu(H_2O, \text{solution})$ decreases, so $\mu(H_2O, \text{crystal}) > \mu(H_2O, \text{solution})$ and there will be a thermodynamic drive to remove water from the crystal. The activation energy for water diffusion is low, so if the process is allowed to proceed, the end result is loss of water with likely deterioration in crystal quality (but see below). Considerations of this kind led Schreuder *et al.* (1988) to develop procedures for solvent modification that would prevent destruction of the crystal. Although some success was reported, sufficient problems were encountered that the approach cannot be considered to be a general solution.

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It is important to note that loss of water does not always lead to loss of crystal integrity. For example, Esnouf *et al.* (1998) and Fu *et al.* (1999) have shown that controlled dehydration can result in substantially improved resolution. In addition, antifreeze concentrations much higher than those needed to suppress ice formation (Mitchell & Garman, 1994) can preserve low mosaic spread. Work by Kriminski *et al.* (2002) suggests these phenomena may be connected.

In earlier work, Travers & Douzou (1970) emphasized the importance of keeping the dielectric constant unchanged when modifying the mother liquor. Petsko (1975) made observations that support the significance of this approach and, based on systematic studies, also showed that keeping $\mu(H^+)$ constant is of great importance. Hui Bon Hoa & Douzou (1973) and Douzou *et al.* (1975) have presented tables of solvent compositions that facilitate the preparation of successful cryoprotective solutions. It should be noted that a significant aim in Petsko's work was to keep the solvent liquid, so as to permit manipulation of enzyme substrates. Studies of enzyme kinetics are much more demanding than the rapid cooling to about 100 K that is of primary interest here.

In most cases it is only necessary to consider kinetic effects, *i.e.*, how long it takes before the crystal itself begins to change. When a crystal in a drop of its original mother liquor is dipped into a drop of modified mother liquor, diffusion begins immediately. The speed of propagation in the liquid phase can be estimated from a standard equation for the mean-square travel distance of a diffusing species,

$$\overline{x^2} = 2Dt,$$

where D is the diffusion coefficient and t is the the time. Typical room-temperature values for D for antifreeze molecules in water are around 10^{-9} m^2 s^{-1}. Thus, a root-mean-square travel distance of 0.1 mm requires about 5 s. For a solvent layer about 0.1–0.2 mm thick, a contact time of 5–20 s should provide a sufficient level of modification to prevent freezing, while the risk of crystal damage is small. It is often important to stop any ongoing process as soon as protection from freezing has been attained. This can conveniently be achieved by immersion in liquid N_2.

10.1.1.2. Internal ice or phase transition

If there are good indications that ice formation does start internally, or that a destructive phase transition takes place, an attempt can be made to modify the internal water structure. An important consideration of Petsko (1975) was never to allow large deviations from equilibrium. This can be accomplished by a slow, gradual change in $\mu(H_2O, \text{solution})$, allowing enough time for the crystal to re-establish equilibrium. A number of successful experiments were reported.

10.1.1.3. Removal of the solvent layer

Because of their tendency toward rapid loss of internal solvent, biocrystals rarely survive prolonged exposure to the atmosphere. A solution to this problem was described by Hope (1988), where the solvent is removed while the crystal is submerged in a hydrocarbon oil. After the liquid has been removed, a small drop of oil is allowed to encapsulate the crystal, allowing it to tolerate brief exposure to air. Even under such mild conditions, some crystals still lose water and suffer damage. A remedy for this is to keep the oil saturated with water. One disadvantage of the oil technique is the tendency of loop mounts to carry along too much oil (Teng, 1990), which can cause excessive background scattering. An advantage is that absorption can become nearly isotropic. The most commonly used oil is the polyisobutene Infineum V8512, formerly known as Infineum Parabar 10312, Exxon Paratone-8277 or Paratone-N. Contrary to popular myth, there is nothing magical or mysterious about this particular oil. Important properties are that it is inert, has a useful viscosity, forms a glass on cooling and has a coefficient of thermal expansion which appears to match that of many biocrystals.

10.1.1.4. Cooling rates

The time dependence of nucleation probability suggests that faster is safer. Although few systematic data are available, it is commonly assumed that crystal cooling should be as rapid as possible. Studies related to cryopreservation of biological samples for electron microscopy provide a number of measurements of cooling rates in various coolants, but it is difficult to extract information directly relevant to cryocrystallography. From a practical point of view, the coolants to be considered are liquid N_2 and liquid propane (and, to a lesser extent, liquid ethane). Thermal conductivities for small-molecule compounds in liquid form tend to be of similar magnitude – around 1.5×10^{-5} W m^{-1} K^{-1}. N_2 boils at 77 K; propane remains liquid between 83 and 228 K. It is often thought that the gas layer that can form around an object dipped in liquid N_2 as a result of the Leidenfrost effect (Leidenfrost, 1756) makes liquid N_2 less effective as a coolant than liquid propane, which is much less likely to form bubbles. However, from model calculations, Bald (1984) suggested that this Leidenfrost insulation problem in liquid N_2 would not be significant in the cooling of small objects of low thermal conductivity, because there is not enough heat transport to the surface to maintain the gas layer. He also concluded that liquid N_2 could potentially yield the highest cooling rate among commonly used coolants, but in a review of plunge-cooling methods, Ryan (1992) gives preference to liquid ethane. Walker *et al.* (1998) measured the cooling rates in N_2 gas (100 K), liquid N_2 (77 K) and liquid propane (100 K) of a bare thermocouple and of a thermocouple coated with RTV silicone cement. The thermocouples were made from 0.125-mm wire and the coating was about 0.20–0.25 mm thick. With the gas stream, cooling of the centres of the samples from 295 to 140 K took 0.8 and 2 s, respectively; with liquid N_2 the times were 0.15 and 0.6 s, and with liquid propane they were 0.15–0.18 and 1.2 s (time reproducibility is to within ±10%). Given the simplicity of liquid-N_2 immersion, there seems little reason to choose the more complicated and more hazardous liquid-propane technique. As the field of low-temperature biocrystallography has matured, liquid-propane methods have all but died out, and liquid-N_2 immersion is now by far the most commonly employed method.

10.1.2. Beneficial effects of low temperature

10.1.2.1. Suppression of radiation damage

Biocrystals near room temperature are sensitive to X-rays and generally suffer radiation damage during data measurement. Often this damage is so rapid and severe that a number of different crystals are needed for a full data set. On occasion, damage is so rapid that data collection is impossible. Crystal decay is typically accompanied by changes in reflection profiles and cell dimensions, which alter the positions of diffraction maxima, exacerbating the problem of changing diffraction intensities. The use of more than one crystal invariably introduces inaccuracies. Intensities from a crystal near the end of its usable