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CRYOCRYSTALLOGRAPHY OF BIOLOGICAL MACROMOLECULES

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X-ray diffraction experiments on crystals of biological macromolecules, especially when carried out on highly intense synchrotron beam lines, frequently suffer from radiation damage. Performing such experiments at cryogenic temperatures virtually eliminates radiation damage thus producing higher quality diffraction data and often making previously intractable problems feasible. In the following article the most important experimental aspects of low temperature diffraction experiments on macromolecular crystals will be discussed.

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1. Introduction

Radiation damage to the crystal caused by the incident X-ray beam has plagued macromolecular crystallography since its early days and, with the advent of ever stronger synchrotron radiation sources, has become the limiting factor in many structural studies on biological macromolecules. Although the photochemical processes producing free radicals (primary radiation damage) are localized events, subsequent chemical reactions can be induced at relatively remote sites due to the possibility that free radicals can propagate in the solvent regions of a protein crystal via diffusion (secondary radiation damage) [1]. Damage is spread and will lead to a loss of crystalline order resulting in poorer quality diffraction data. It was noted as early as 1970 [2] that performing diffraction experiments on proteins at temperatures lower than room temperature leads to a significant reduction in radiation damage. This effect is due to the fact that by lowering the temperature diffusional processes and therefore propagation of highly reactive agents within crystals are slowed down. Going to temperatures around 100 K at which diffusion has come to a complete halt on the time scale of a typical experiment should therefore virtually eliminate secondary radiation damage (see Fig. 1). Primary radiation damage will still occur, but in fact only represents a problem for extremely weakly diffracting systems [3].

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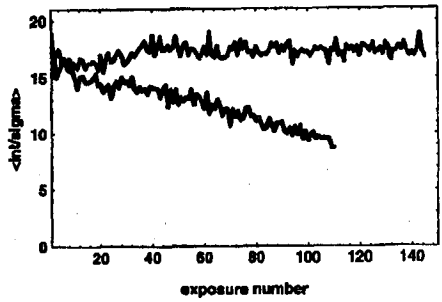

Fig. 1. Ratio of $\langle I/\sigma(I)\rangle$ (I and $\sigma(I)$ being intensities and estimated standard deviations of the intensities, respectively) for consecutive 1° oscillation photographs taken from a triclinic crystal of lysozyme. Data were collected at 300 K (black line) and 120 K (grey line) on beam line BW7B and beam line X11 at EMBL c/o DESY, Hamburg, using exposure times of $\approx$ 1 min and $\approx$ 2 min per image respectively.

Although the benefits of working at low temperatures have been appreciated by many small molecule crystallographers for decades, protein crystallographers have only started to make extensive use of cryogenic techniques relatively recently [4]. This is mainly due to the difficulties encountered when protein crystals are cooled to temperatures below the freezing point of their buffer.

In this article the basic experimental setup for a diffraction experiment at cryogenic temperatures is described first. Then the principles of pretreating crystals for successful shock-cooling ("cryoprotection"), crystal mounting and cooling are introduced. Finally, the particular benefits of using cryotechniques in conjunction with synchrotron sources are briefly discussed.

Reviews on cryogenic techniques in macromolecular cryocrystallography can be found in Refs. [5–8]. The book by Rudman [9] provides an excellent overview of the use of cryogenic techniques in small molecule crystallography. Larsen [10] has recently given an overview of diffraction studies below 77 K. Useful information can also be found on the World Wide Web (keywords "cryocrystallography" or "flash cooling" using any search engine).

2. Experimental setup

A schematic view of a typical experimental setup is given in Fig. 2. Instead of being sealed in a capillary tube, the crystal is suspended by a thin film in a loop [11] made of a thin fibre such as single threads of dental floss, wool or rayon. The temperature of the crystal is maintained by a stream of nitrogen gas at the required temperature. To avoid the formation of ice around the crystal, the nitrogen stream is shielded against humidity contained in the surrounding atmosphere by a coaxial stream of warm dry air or nitrogen. For complete elimination of ice build-up during experiments over extended periods of time the relevant parts should be enclosed e.g. by a box made of plexi-glass or a tent built from plastic film. Such enclosures not only prevent any humidity from coming close to the cold parts but also shield the experiment from drafts which could cause turbulence in the gas streams. If necessary entire diffractometers can be put into a dry box.

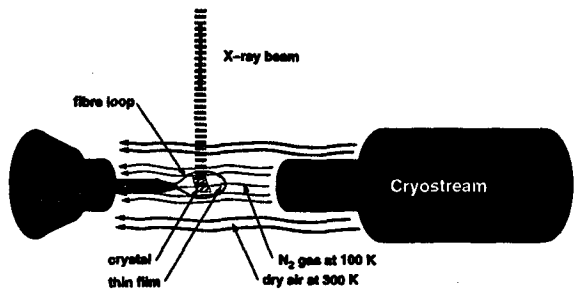

Fig. 2. Schematic view of a diffraction experiment at cryogenic temperatures.

3. Cryoprotection of crystals

When a crystal of a biological molecule is cooled to cryogenic temperatures the main difficulty is to avoid the formation of crystalline ice within the sample and in the liquid on its surface: the 9% increase in specific volume during the phase transition from water to ice invariably disrupts the crystal lattice. Therefore a cooling procedure has to be chosen which avoids the formation of ice and instead leads to a glass-like amorphous phase of the solvent ("vitrification") [12]. In principle there are three options: (1) cooling on a time scale too fast for ice formation to occur [13], (2) cooling at high pressure by which the formation of the common hexagonal form of ice can be circumvented [14], (3) modifying the physicochemical properties of the solvent in and around the crystal by addition of solutes ("cryoprotectants") in a way that the vitrified state can be reached at moderate cooling rates [12]. The latter method is currently the most widely used, since very fast cooling in most cases involves the use of potentially dangerous cryogens such as liquid propane or ethane and the high-pressure method requires elaborate equipment.

Addition of organic solutes to the stabilizing solutions used for protein crystals lowers the freezing point of the respective solutions and slows down the crystallization kinetics of ice [15]. The first condition for a cryoprotective buffer is that the buffer should, at a given cooling rate, not show any detectable crystalline ice. This can be easily checked by cooling small amounts of a buffer in the cold nitrogen stream: if the drop or film stays transparent upon cooling, an amorphous glass has been formed, if it becomes opaque ice crystallites have grown in the sample. A large number of compounds [7, 8] have been employed as anti-freeze agents, but in many cases the strategy outlined in Table will lead to success. If MPD (2-methyl-2,4-pentanediol) or PEG's (polyethyleneglycols) of molecular weight below 4000 have been used as the precipitant, increased concentrations of these compounds should be examined first. For PEG's of molecular weight larger or equal to 4000, small PEG's (PEG-400 or PEG-600) will work in many cases. In all other cases, except for salt-induced crystals, glycerol in concentrations from 10 to 40% will be a promising candidate. Crystals grown from salt-rich solution are frequently difficult to cryoprotect due to the limited solubility of many salts in aqueous/organic mixtures. At low salt concentrations MPD and ethylene glycol

TABLE

Strategy for choice of cryoprotectants for systems where organic compounds or salts at relatively low concentrations were used as precipitating agents.

Precipitant	Cryoprotectant
MPD	MPD
PEG < 4000	precipitant
PEG ≥ 4000	PEG-400, PEG-600
low salt	MPD, ethylene glycol
all other	glycerol

have successfully been used, but at high salt concentrations an exchange of the salt prior to any cryoexperiments might be necessary [16, 17].

Generally cooling conditions and procedures should be optimized to minimize adverse effects on the crystals to obtain data of the highest possible quality. Parameters to be varied include the choice of the anti-freeze agent and its concentration but also the method by which crystals are transferred from their stabilizing solution to the cryoprotective buffer. Differences in osmotic pressure frequently lead to cracking or complete dissolution of crystals. A stepwise transfer via buffers containing increasing concentrations of the cryoprotectant or dialysis against the cryoprotective buffer can help to minimize such effects. Another possibility is to crystallize in the presence of cryoprotectants.

4. Crystal mounting and data collection

To facilitate rapid heat transfer the crystal has to be in immediate contact with the cooling medium and therefore capillaries cannot be used for mounting. Depending on the mechanical properties of the crystal, glass fibres [4, 18], glass spatulas [19] or fibre loops [11] can be used. Here we will concentrate on the loop method. After the crystal has been equilibrated with a cryoprotective buffer a loop of appropriate size is selected and used to detach the crystal from the surface of the well (see Fig. 3). Gentle stirring of the liquid in an upward motion will bring the crystal up to the meniscus of the liquid. The crystal can then be picked up by swiftly moving the loop alongside the crystal. To obtain a thin film it is necessary to move the loop with its plane perpendicular to the surface of the drop. The crystal will be held within the film by surface tension and must now be cooled to cryogenic temperatures as quickly as possible (ideally the transfer time should

Fig. 3. Fishing a crystal using a loop.

be less than one second). In most cases cooling in the nitrogen stream ("stream freezing") is sufficient, but sometimes (e.g. if crystals are larger than 0.4 mm in the longest dimension) other cooling procedures such as propane plunging [12, 13] might be required. Once the crystal is in place, data collection can proceed as usual.

If against all precautions ice builds up during the data collection, powder diffraction rings will appear on the diffraction patterns. Depending on the amount of ice it may be advisable not to take any risk by removing the ice, but to deal with the additional diffraction in the data processing stage by using appropriate rejection criteria.

5. Storage and transport of crystals

Once a crystal has been successfully cooled to cryogenic temperature it can in principle be stored for indefinite times. This allows to cool and characterize crystals "when they are best" on a conventional source in the home laboratory and then store them at cryogenic temperatures until synchrotron time becomes available. Dewars which can be used for transport, including shipment by airplane, are commercially available. The possibility of characterizing crystals at home and the much safer transport of crystals at cryogenic temperatures makes data collection at synchrotrons far more predictible and efficient.

6. Conclusion and perspectives

Cryogenic methods offer great advantages in macromolecular crystallography especially when synchrotron radiation is used for diffraction data collection. Most of the difficulties are technical in nature and can be overcome. Apart from alleviating the problem of radiation damage and enabling storage and safe transport of crystals, there are a number of further benefits. In general, higher data quality can be achieved and in many cases all necessary data can be collected from a single crystal. This is of special importance when multiple anomalous dispersion (MAD) methods are applied for phasing [20, 21] and in studies aimed at atomic resolution data [22]. Multiple temperature studies allow the investigation of protein dynamics by crystallographic methods [13, 23] and many important insights into how proteins work will arise from experiments on different redox states [24] and intermediate stages of reactions [25] which can be stabilized and characterized in crystals by using cryogenic methods.

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