microbatch under oil, microdialysis, and free interface diffusion could be used for setting up crystallization experiments. The most frequently used crystallization method is the *vapor diffusion technique*. The difference in concentration between the drop (protein, buffer, salt and precipitant) and the reservoir (buffer, salt and precipitant) drives the system toward equilibrium by diffusion through the vapor phase. The protein becomes supersaturated and crystals start to form when the drop and reservoir are at or close to equilibrium. *Free interface diffusion* is one of the methods used by NASA in microgravity crystallization trials. Using this method the sample is in liquid contact with the precipitant. Over time the sample and precipitant diffuse into one another and crystallization may occur at the interface. *Batch crystallization* is method where the sample is mixed with the precipitant and additives creating a homogenous crystallization medium. This technique is popular with small molecule crystallographers. In *microbatch under oil* a small drop of the sample combined with the crystallization agent is pipetted under a layer of oil (paraffin, silicon oils). Such oils allow water vapor to permeate from the drop and allow sample and reagent concentration. Unless the drop is equilibrated with a reservoir, water will leave the drop until those only solids remain. *Dialysis crystallization* involves placing the sample in a Dialysis Button, which is sealed with a dialysis membrane. The Dialysis Button is placed into a container with crystallization medium. Water and some precipitants are then allowed to exchange while retaining the sample in the cell.

For the **examination** of the crystallization trials a stereomicroscope is used. Crystals are usually easy to distinguish from amorphous precipitate. Diffractable crystals are typically single, transparent, they have definite form characterized by planar faces and they are free of cracks and defects. Crystals are often birefringent, so that they appear dark and bright as they are rotated under crossed polarizers in the stereomicroscope. Several methods are available to test whether crystals are protein or salt. These are crush test, dehydration test, dye binding test, gel electrophoresis and X-ray diffraction. In the case of getting microcrystals, the seeding techniques could be used to grow the crystal. The seeds (microcrystals) are transferred to a new protein-precipitant drop using a streak seeding wand or a crystal transfer syringe, respectively. Seeds provide a template on which further molecules can assemble, and given the proper environment, time, and patience, the seed will enlarge into a crystal.

The crystallization of membrane proteins proceeds in the same manner as crystallization of soluble proteins, except for the addition of detergents in the crystallization conditions. Selection of proper detergent is the most critical parameter for this kind of crystallization.

Experience and reproducibility are guides in making crystallization experiments.

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CRYOCRYSTALLOGRAPHY OF BIOLOGICAL MACROMOLECULES

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Keywords

cryocrystallography, radiation damage, protein crystals, cryoprotectants

Abstract

Crystals of biological macromolecules at near to room temperature are sensitive to X-rays and frequently suffer from radiation damage, especially when X-ray experiments are carried out on highly intense synchrotron beamlines. Performing such experiments at cryogenic temperatures greatly reduce, or eliminate radiation damage and thus produce higher quality diffraction data. Since about 1985, the cryocrystallography methods have become widely used and well established technique. A brief discussion of the most important experimental aspects and advantages of data collection at low temperature is given. Reviews on cryogenic techniques in macromolecular crystallography can be found in: [1, 2, 3].

Radiation damage

Radiation damage of biocrystals appears to be related to the formation of free radicals. Although the photochemical processes producing free radicals (primary radiation damage) are localized event; subsequent chemical reactions can be induced at relatively remote sites due to the propagation of free radicals in the solvent regions of a protein crystal via diffusion (secondary radiation damage) [4]. Damage is spread and leads to crystal decay, typically accompanied by changes in reflection profiles and cell dimensions.

It was noted as early as 1970 [5] that performing diffraction experiments on protein crystals cooled to near liquid-N_2 temperature leads to significant reduction in radiation damage. This effect is due to facts that by lowering the temperature diffusional processes and therefore propagation of higly reactive free radicals within the crystals is slowed down.

Experimental setup

A schematic of a typical experimental setup of a diffraction experiment at cryogenics temperature is shown in Figure 1. Instead of being sealed in capillary, the crystal is mounted in a thin nylon fiber loop and rapidly cooled, either in a cold gas stream, or by immersion in a cryogen such as liquid nitrogen. The temperature of the crystal is maintained by a stream of nitrogen gas during the diffraction measurement. To avoid the formation of ice around the crystal, the nitrogen stream is shielded agains humidity by a coaxial stream of warm dry air.

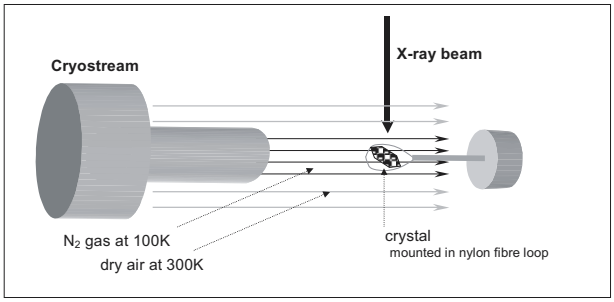

Figure 1. Schematic view of a typical experimental setup of diffraction experiment at cryogenics temperature

Cryoprotection of crystals

When a crystal of biological macromolecule is cooled to cryogenic temperature the main difficulty is to avoid the crystallization of any water present in the system, whether internal or external to the crystal. Therefore a cooling procedure has to be chosen that leads to a glass-like amorphous phase of the solvent [6]. In principle, there are three options: (1) cooling on the timescale too fast for ice formation to occur [7], (2) cooling at high pressure by which the formation of common hexagonal form of ice is circumvented [8], (3) modifying the physicochemical properties of the solvent by addition of cryoprotectants in a way that vitrified state can be reached at moderate cooling rates. The latter method is currently the most widely used. A list of cryoprotectants used successfully with macromolecular crystals is shown in Table 1 [9]. Glycerol appears to be a widely applicable cryoprotectant and is frequently chosen for initial trials [10]. Typically cryoprotectants are included in the established harvesting solution at concentration range 50%. Methods for introducing the cryoprotectants are: (1) serial transfer into increasing concentrations of cryoprotectant, (2) dialysis, (3) growth in cryoprotectant, (4) brief transfer before flash cooling, (4) direct transfer into full strenght cryoprotectant. It is rare that crystal can be transferred without damage directly to a solution containing full-strenght cryosolvent. Usually, the cryoprotectant must be introduced slowly to reduce stress on the crystal lattice. 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 must be minimized.

Crystal mounting and data collection

To facilitate rapid heat transfer the crystal must 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, glass spatulas or, currently most widely fibre loops are used [11]. Using the nylon fibre loop, protein crystal is picked up by swiftly moving the loop alongside the crystal from the crystallization mother liquor. The crystal is held within the film by surface tension and after equilibration in cryoprotective buffer must be cooled to cryogenic conditions as soon as possible. A simple and often effective approach is to flash cool the crystal in a goniostat nitrogen stream right on the X-ray camera. This technique has the added advantage in leaving the crystal in position for immediate analysis and data collection. An alternative method, rapidly plunging the crystal into a liquid cryogen, also offers several advantages. It reduces the time between mounting the crystal and flash cooling, it produces higher cooling rate and results in more even cooling of both sides of loop-mounted sample. Crystal flash cooled in a liquid nitrogen must be placed for data collection in the cold gas stream a goniostat without any substantial warming.

Storage and transport of crystals

Once a crystal has been successfully cooled to cryogenic temperature it can be in principle stored for indefinite time. This allows to cool and characterize crystals on a conventional source in the home laboratory and then store them until synchrotron time becomes available. Dewars that can be used for transport, including shipment by airplane, are available.

Conclusion and perspectives

Cryogenic methods provide great advantages in macromolecular crystallography especially when synchrotrone radiation is used for diffraction data collection. Apart from eliminating the problem with radiation damage and enabling the storage and safe transport of frozen crystals, there are number of additional benefits. In general, higher quality data can be achieved and in many cases all data can be collected from a single crystal. Cryogenic data collection has allowed efficient phasing using multiwavelength methods. Additionally, a crystal at cryotemperature is rigidly attached to its mount, so that slippage during the measurement is impossible.

List of cryoprotectans used successfully for flash cooling of biological crystals	
Erythriol	2-Metyl-2, 4-pentanediol (MPD)
Ethanol	Polyethylen glycol 400
Ethylen glycol	Polyethylen glycol 1000 – 10 000
Glucose	Propylene glycol
Glycerol	Sucrose
Methanol	Xylitol

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X-ray protein diffractometer and basis of the rotation method

PROTEINOVÝ DIFRAKTOMETR – MĚŘENÍ ROTAČNÍ METODOU

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Keywords:

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Abstract

X-ray data collection is central experiment in a crystal structure analysis. Recent advances such as cryogenic cooling and two-dimensional detectors have made data collection technically easier, but it remains fundamental scientific procedure underpinning the whole structural analysis. A brief discussion of the most important aspects of the data collection is given. Reviews on X-ray data collection techniques in macromolecular crystallography can be found in: International Tables for Crystallography, *Volume F,* 177 - 195

Úvod

Měření difrakčních dat je základní experiment v krystalové strukturní analýze. Pro struktury malých molekul jsou běžně měřena data do atomového rozlišení (většinou do 0.8 Å), takže fázový problém může být řešen přímo a pozice atomů mohou být rafinovány plně anizotropním modelem.

Krystaly makromolekul jsou z hlediska měření difrakčních dat mnohem problematičtější. První problém vyplývá z velikosti elementární buňky, díky které jsou průměrné intenzity reflexí mnohem nižší a jejich počet vyšší (tabulka 1). Za druhé, část objemu krystalu tvoří kanály vyplněné neuspořádaným rozpouštědlem, takže dále snižují intenzitu reflexí na vysokém rozlišení a ve většině případů omezují rozlišení na mnohem nižší než atomové. Za třetí, opět především díky obsahu rozpouštědla, jsou krystaly citlivé na radiační poškození. Nedávný technický pokrok v měřících metodách, jako např. používání synchrotronového záření, měření zmražených krystalů a vysoce účinné plošné detektory, usnadnil měření, ale to stále zůstává základem celé strukturní analýzy. Proto je nezbytné tomuto klíčovému kroku věnovat maximální pozornost.

1. Součásti proteinového difraktometru

Tabulka 1. Velikost elementární buňky a počet reflexí.

Sloučenina	Mříťový parametr [Å]	Objem buňky [Å]	Počet reflexí	Prům. intenzita
Organická sl.	10	1000	2000	1
Supermolekula	30	27000	30000	1/27000
Protein	100	10^6	10^5	10^{-6}
Virus	400	10^8	10^6	10^{-8}

Pro sběr dat z makromolekulárního monokrystalu při konkrétní vlnové délce potřebujete difraktometr složený z následujících součástí:
(1) zdroj RTG záření
(2) optickou soustavu pro fokusaci RTG záření na vzorek
(3) monochromátor pro selekci jedné vlnové délky záření
(4) kolimátor pro úpravu rozměrů RTG záření
(5) „závěrku“ (shuter) pro kontrolu expozice vzorku
(6) goniostat spojený s držákem vzorku

Dále je velice vhodné mít k dispozici:
(1) zařízení pro měření zmražených krystalů
(2) účinný plošný detektor
(3) software pro měření, ukládání a zobrazování exponovaných dat
(4) software pro zpracování dat, tj. získání intenzit jednotlivých Braggovských difrakcí a jejich chyb.

2. Zdroj RTG záření - rotační anoda

Ve strukturní analýze makromolekul je praktiky vždy používána Cu anoda s fixní vlnovou délkou 1.542 Å (pokud není zdrojem synchrotronové záření). Rotační anoda má proti klasické rentgence výhodu vyšší intenzity RTG záření. Systémy jsou dnes běžně vybaveny grafitovým monochromátorem, nebo fokusačními zrcadly, nebo vícevrstvou optikou (Osmic), které zajišťují fokusaci