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Efficient cryoprotection of macromolecular crystals using vapor diffusion of volatile alcohols

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ABSTRACT

Macromolecular X-ray crystallography, usually done at cryogenic temperature to limit radiation damage, often requires liquid cryoprotective soaking that can be labor intensive and damaging to crystals. Here we describe a method for cryoprotection that uses vapor diffusion of volatile cryoprotective agents into loop-mounted crystals. The crystal is mounted into a vial containing a small volume of an alcohol-based cryosolution. After a short incubation with the looped crystal sitting in the cryosolution vapor, the crystal is transferred directly from the vial into the cooling medium. Effective for several different protein crystals, the approach obviates the need for liquid soaking and opens up a heretofore underutilized class of cryoprotective agents for macromolecular crystallography.

1. Introduction

Data collection at cryogenic temperature has become the normal approach for structure determination via X-ray diffraction. The low temperature (typically 100 K) slows radiation damage and is especially useful at high intensity synchrotron radiation sources (Kmetko et al., 2006; Owen et al., 2006). However, cryogenic cooling itself can damage the crystal and compromise diffraction quality, often due to ice formation (Haas and Rossmann, 1970; Juers and Matthews, 2001, 2004; Kriminski et al., 2002; Low et al., 1966). Cooling-induced damage is typically reduced by cooling faster and/or adding cryoprotective agents such that the system cools through the freezing point of water to the glass transition before ice can form (Chinte et al., 2005; Shah et al., 2011; Warkentin et al., 2013). The use of pressure to prevent the formation of ice I during cooling has also been successfully applied to several systems (Burkhardt et al., 2012; Kim et al., 2005; Thomanek et al., 1973).

Many different cryoprotective agents have been identified (Bujacz et al., 2010; Gulick et al., 2002; Holyoak et al., 2003; Hope, 1988; Marshall et al., 2012; Mueller-Dieckmann et al., 2011; Pemberton et al., 2012; Rubinson et al., 2000; Vera and Stura, 2014), including sugars, linear and branched polyols, salts, organic solvents, amino acids, methylamine osmolytes and viscous hydrocarbons. In some cases, an adequate cryoprotective agent is already present in the crystallization buffer and the crystal can be cryocooled directly from the growth drop. However, very often an additional cryoprotection step is performed by soaking the crystal in a cryosolution, which can be laborious and damaging to crystals due to handling and osmotic stresses. Approaches to cryoprotection that limit such treatments would be advantageous.

Volatile alcohols are known to be efficient cryoprotective agents and have been useful for cryopreservation of microorganisms (Hubalek, 2003) and for low temperature crystallography in the liquid state (Douzou et al., 1975). Recent experiments showed that both methanol and ethanol require lower concentrations (w/v) than traditional cryoprotective agents (e.g. glycerol and ethylene glycol) to prevent ice formation in small volumes of plunge-cooled solution (Warkentin et al., 2013). Despite their effectiveness, volatile alcohols have seen little use for cryoprotection in macromolecular crystallography, due in part to the difficulty of working with their high vapor pressures. Of the ~100,000 structures in the protein data bank, just 0.2% have methanol or ethanol present in the model, while 14% include either glycerol or ethylene glycol (Berman et al., 2000). A recently described vial mounting method

Abbreviations: DMSO, dimethyl sulfoxide; DMF, dimethylformamide; $AmSO_4$, ammonium sulfate; NaOAc, sodium acetate; PEG, polyethylene glycol; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; MeOH, methanol; EtOH, ethanol; iPrOH, isopropanol; tBuOH, t-butanol.

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offers the possibility of turning the high vapor pressure into an advantage to deliver the volatile alcohol to a loop-mounted crystal (Farley and Juers, 2014). Here we show the approach is rapid and effective for several different protein crystals. Subsequent cryocooling yields high quality diffraction without ice formation. The approach does not require liquid soaking and opens up a new class of cryoprotective agents for macromolecular crystallography.

2. Materials and methods

2.1. Crystals

Chemicals were from Hampton Research (Aliso Viejo, California, USA; glucose isomerase #HR7–100) or Sigma–Aldrich (St. Louis, Missouri, USA; all other chemicals). Orthorhombic glucose isomerase crystals were used as provided by the supplier. All other crystals were grown using hanging drop vapor diffusion with 24 well plates (Hampton Research, Aliso Viejo, CA) at 294–298 K (277 K for hexagonal thaumatin) and used within a few months of growth. Tetragonal lysozyme (#L6876) well: 20 mM NaOAc 4.5, 3–5% w/v NaCl; protein: 80–100 mg/mL in 20 mM NaOAc 4.5 (Forsythe et al., 1999). Orthorhombic and trigonal trypsin (#T8003) well: 100 mM Tris 8.0, 25% w/v PEG 8000, 0.2 M $AmSO_4$, 0.1 M benzamidine HCl; protein: 50 mg/mL in water (Leiros et al., 2001); Tetragonal thaumatin (#T7638) well: 0.2 M–0.9 M Na/K tartrate; protein: 35–70 mg/mL in 100 mM HEPES 7.3 (Ko et al., 1994). Hexagonal thaumatin (#T7638) well: 0.1 M NaOAc 4.5, 0.175 M $AmSO_4$, 0.1 M $LiSO_4$, 0.1 M $MgCl_2$, 15% (v/v) glycerol, 2% (w/v) PEG 400; protein: 35 mg/mL in 100 mM HEPES 7.3 (Charron et al., 2004); Thermolysin (#P1512) well: 30% sat'd $AmSO_4$; protein: 150 mg/mL in 45% v/v DMSO (hexagonal); 100 mg/mL in 45% v/v DMSO, 0.5 M $ZnCl_2$ (tetragonal) (Hausrath and Matthews, 2002). Tetragonal proteinase K (#P6556) well: 0.3–0.4 M Na/K tartrate or 12–15% w/v PEG 8000; protein: 30–50 mg/mL in water. Cubic insulin (#I5523) well: 345–525 mM NaPhosphate dibasic, 10 mM EDTA 9.2; protein: 15 mg/mL in 18 mM NaPhosphate dibasic, 10 mM EDTA 10.5 (Gursky et al., 1992). In all cases, drop sizes were 6–9 μL and were ½ well/½ protein, except for thermolysin, which used just the protein solution given set up over the well. Prior to cryocooling, some thermolysin crystals were serial diluted (2–3 min) into DMSO-free protein solution (i.e. water for hexagonal crystals and 0.5 M $ZnCl_2$ for tetragonal crystals) to ensure the absence of the natural cryoprotective effects of DMSO. Similarly, some glucose isomerase crystals were serial diluted over 2–3 min from their 0.9 M $AmSO_4$ solution into 0.25 M $AmSO_4$.

2.2. Cryosolutions

Cryosolutions were based on four volatile alcohols – methanol, ethanol, isopropanol, and tert-butanol. Binary cryosolutions (alcohol/water) were prepared gravimetrically, while well-based cryosolutions were prepared volumetrically using 2× well solution, water and the alcohol. Because the low surface tension can make vial mounting difficult (see below), we also tested a cryosolution of 7.5% agar, 40% methanol and 52.5% water (by weight). The agar was dissolved in hot water and pipetted into a cryovial. Then the methanol was added and the solution was mixed, covered with a crystal-cap and O-ring and allowed to cool.

2.3. Vial mounting and cryoprotection

Vial mounting proceeded as previously reported (Farley and Juers, 2014). Briefly, a cryovial (Hampton Research, Aliso Viejo, California, USA) was prepared by plugging the liquid nitrogen escape holes with clay and fitting an O-ring (amazon.com, nitrile rubber, 50A durometer hardness; 3/8″ ID × 1/16″ thick) on the crystal cap (SPINE, Hampton Research). Crystals were mounted by placing the crystal growth coverslip in a humid flow of 85–98% RH, looping the crystal using cryoloops of 20 μm diameter nylon with microtubes snapped at the 18 mm notch (Hampton Research) and inserting into a vial containing 500 μL of cryosolution. Crystals were mounted directly from drops without adding extra solution. (Sometimes crystals were pushed into the drop.) The vial was allowed to sit for some time period (a few seconds up to 16 h). Our default condition was 2 min equilibration against 40% w/w methanol. After equilibrating, the crystal was directly mounted on the diffractometer from the vial. It is recommended that the vial undergo minimal handling and that the crystal cap be manipulated with a thermally insulated wand in order to uniformly maintain the cap-vial system at ambient temperature. The vial mounting technique should be practiced to achieve the smooth motions required to prevent crystals from being dislodged from the loop. The goniometer should be positioned such that the vial is at least horizontal and ideally angled downward as it is removed from the crystal cap, keeping the low surface tension cryosolution towards the bottom of the vial. The cryosolution can also be prepared as an agar gel to limit its movement during mounting (see above).

2.4. X-ray data collection

X-ray data were collected using an Agilent Xcalibur X-ray diffractometer with a Nova X-ray source and Onyx detector (Agilent Technologies, Santa Clara, California, USA) using the following parameters: 50 kV, 0.8 mA, crystal to detector distance = 65.000 mm, theta (the detector angle) = 3.5°, oscillation width = 0.25°, number of frames: 2 × 6, separated by 90°. The detector edge was set to 1.8 Å for all crystals, regardless of their diffraction power. Exposure times were 15 or 30 s, the latter if the shorter exposure did not yield 2.0 Å data. Data were processed with CrysalisPro (Agilent) in Pre-experiment mode, which outputs cell parameters, an estimate of the diffraction limit and the mosaicity.

3. Results and discussion

Fig. 1 compares diffraction images from crystals incubated in-vial over crystal growth well solution vs an alcohol-based cryosolution. The crystals equilibrated over alcohols show high quality diffraction to at least 2.0 Å resolution, comparable to crystals cryoprotected by soaking in traditional cryoprotectants (i.e. ethylene glycol, glucose, MPD) while the negative controls show ice and reduced diffraction power. Many of the crystals diffracted to much higher resolution than 2.0 Å, and data sets were collected to 0.95, 1.3, 1.5, 1.5, 1.5, 1.8, and 1.9 Å resolution for trypsin (orthorhombic), proteinase K, lysozyme, glucose isomerase, thaumatin (tetragonal), thermolysin (tetragonal) and insulin, respectively. The approach was effective for eliminating ice from well-diffracting crystals as well as the complete cryoprotection of crystals for which the negative control destroyed the crystal lattice. Initially, in-vial equilibration times of tens of minutes were used, since we found previously that small unit cell changes occur on that time scale for vial mounts of thaumatin crystals (Farley and Juers, 2014). Subsequently, for most of the proteins we tested shorter equilibrations (except for trigonal trypsin for which we only had two crystals) finding 10 s–3 min produced high quality diffraction.

The method was successful with all ten crystals tested. Eight crystals could be cryoprotected using the vapor of a simple binary solution of water and alcohol and two crystals required supplementing the crystal growth solution with alcohol. For tetragonal