Introduction to single crystal X-ray analysis XII. Tips for collection and processing of protein crystal data

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1. Introduction

Single crystal X-ray analysis of proteins involves many hurdles to be overcome, including protein expression, crystallization, data collection and phase determination. Advanced radiation facilities, innovative detector systems, laboratory systems with high-intensity X-ray sources and newly developed software have drastically expanded the range of crystals that can be analyzed. Despite such improvements, there are still some hurdles to be overcome, i.e., quality checking of protein crystals and optimization of freezing conditions. No matter how good device or software may be, crystals of poor quality cannot yield any data suitable for structural analysis. The quality of crystals can deteriorate when frozen under non-optimized conditions, due to crystallization of water contained in the crystals. Even if crystals produce diffraction spots at sufficient resolution for analysis, if any ice rings are present in the image, then the diffraction spots within the ice ring-containing regions need to be excluded, resulting in poor quality and completeness of the data. This manuscript provides some tips for collecting and processing protein crystal data for X-ray analysis.

2. Screening of crystals

The quality of crystals cannot be assessed merely by microscopic observation; it can only be assessed by exposing them to X-rays and checking the actual diffraction spots and resolution. A perfect-looking crystal with a clear shape may produce hardly any diffraction spots or distorted spots upon X-ray exposure. On the other hand, a rounded, irregularly shaped crystal may be able to produce diffraction spots at high angles and provide analyzable data. Moreover, crystals from the same crystallization batch do not always have the same quality; one may produce elongated diffraction spots or be unable to produce spots at high angles, while an adjacently formed crystal may yield well-defined spots at high resolution. Thus, screening of crystals should be performed by using multiple crystals instead of using only one crystal. Generally, crystals are individually picked up and subjected to freezing, measurement and evaluation, after which only those of high quality are subjected to the main measurement or used in radiation measurement. However, this approach greatly depends on the experimenters' skills and cannot avoid the risk of quality deterioration of crystals during their pick-up or freezing. By using a PX Scanner (Figure 1) or PlateMate (Figure 2), the crystals can be analyzed in situ within crystallization plates to eliminate the risks of quality deterioration over time upon pick-up or damage caused by freezing.

Fig. 1. *In situ* X-ray diffraction screening system PX Scanner.

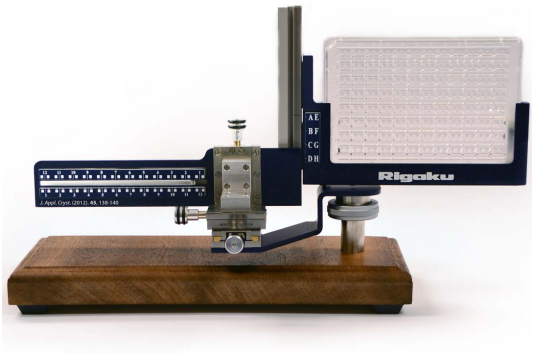

Fig. 2. Crystallization plate adapter for *in situ* X-ray irradiation PlateMate.

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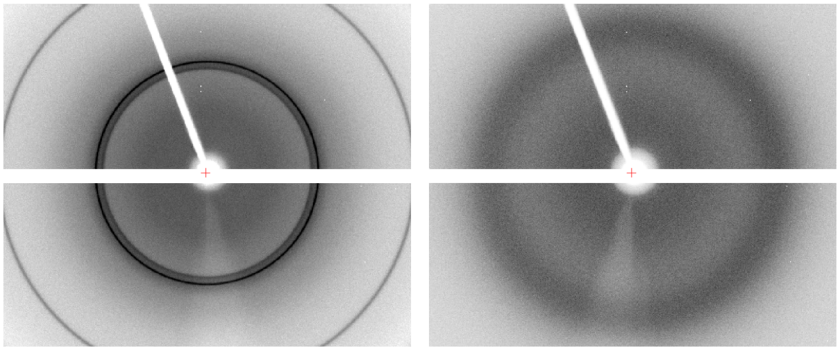

Fig. 3. Left: An example image with an ice ring; Right: An image obtained using an appropriate cryoprotectant.

3. Freezing of protein crystals

Currently, protein crystals are generally analyzed in frozen states. However, since protein crystals contain many solvent regions, most protein crystals, when frozen directly, suffer crystallization of water contained therein. Water in the form of hexagonal crystals (ice) has a larger volume compared to its liquid state, causing quality deterioration or collapse of the protein crystals. Formation of numerous ice crystals leads to ring-like diffraction patterns called "ice rings", and the regions containing these ice rings must be excluded for the analysis. No matter how high quality of the obtained crystals may be, any failure in the freezing process could lead to quality deterioration and spoil the entire experiment. Thus, screening of freezing conditions is a very important step. Formation of ice can be suppressed by using cryoprotectants. When protein solutions containing sufficient amounts of cryoprotectants are rapidly frozen, water vitrifies instead of forming crystals. This enables freezing of protein crystals without damaging them while suppressing ice-ring formation. Commonly used cryoprotectants are glycerol, ethylene glycol and polyethylene glycol.

3.1. Screening of freezing conditions

As a preliminary experiment, add cryoprotectants to the crystallization solvents and freeze the solvents to see whether they remain transparent, as water remains transparent when vitrified. Frozen solvents that appear to be transparent may contain tiny water crystals, so they need to be checked by applying X-ray beams to confirm the absence of ice rings. When the solvents turn white upon freezing or ice rings are observed, the cryoprotectants need to be added at higher concentrations (Figure 3). In cases where polyethylene glycol or ethylene glycol is used for crystallization, increasing their concentrations may improve the freezing outcomes.

Once the optimum freezing conditions are determined by the preliminary experiments, pick some protein crystals that are too small to be used for the main measurements and soak them in the cryoprotectants to see whether they dissolve or crack in these agents. To avoid the risk of wasting the crystals that can be used for the main measurements, it is recommended to first soak smaller crystals in the cryoprotectants to check the absence of cracking or any other damage. If no damage is observed, then freeze the larger crystals and perform the main measurement. If smaller protein crystals dissolve or crack, try again with increased concentrations of the cryoprotectants and the precipitants or with different cryoprotectants.

3.2. Freezing methods

Methods for freezing protein crystals can be roughly divided into two types: methods using cryogenic gas streams from low temperature system and those using liquid nitrogen.

In methods using cryogenic gas streams, the cryogenic gas streams are blocked with name cards, etc., while mounting the loops containing the protein crystals. After the loops are mounted, the name cards are removed to allow flash freezing (Figure 4).

In methods using liquid nitrogen, the crystal-containing loops are rapidly soaked in liquid nitrogen. After the liquid nitrogen stops boiling, the loops are mounted onto goniometer heads using tongs (Figure 5).

4. Data collection

For data collection, S/N can be improved by employing smaller oscillation angles per image and shorter exposure times for the measurement. Smaller oscillation angles are also preferable for improving resolution of individual diffraction spots produced by protein crystals having long lattice parameters. With laboratory-scale devices, measurements are typically performed using oscillation angles of 0.25–0.5°per image.

Longer exposure times are effective for measuring the diffraction spots at higher angles but may excessively increase the intensities of spots at lower angles to cause their saturation. This can be prevented by using different 2θ for performing the low- and high-angle measurements and adopting short and long exposure times at low- and high-angles, respectively, which should enable collection of data with higher resolution.

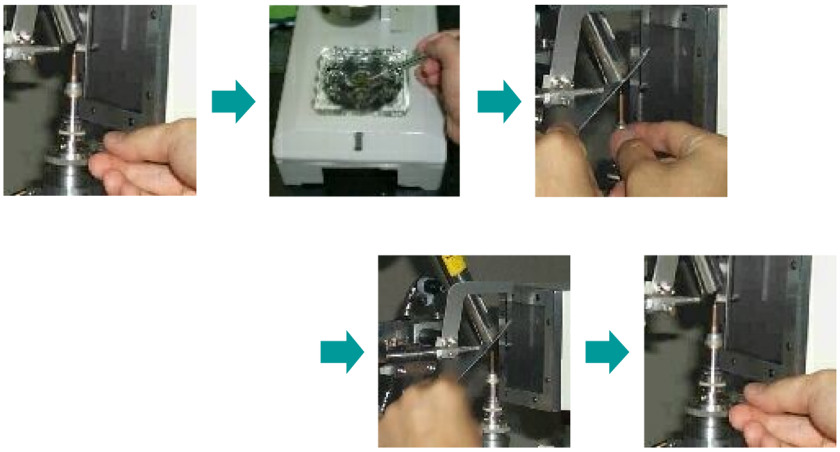

Fig. 4. Freezing procedures using a cryogenic gas stream.

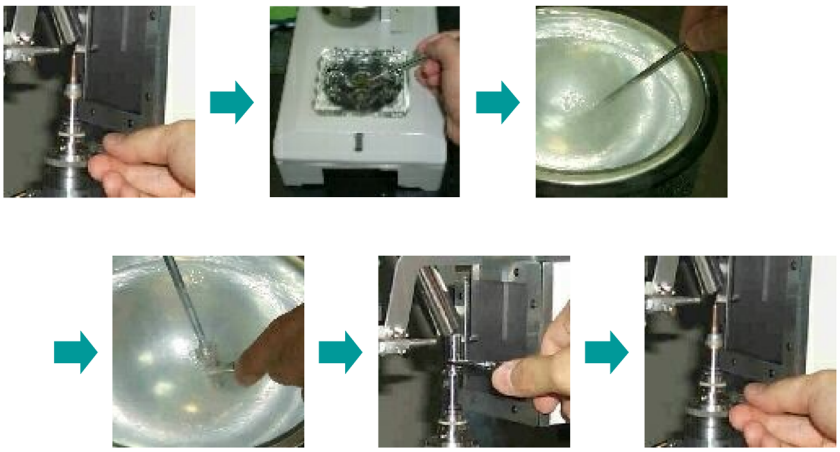

Fig. 5. Freezing procedures using liquid nitrogen.

5. Data processing

Check the Rmerge, completeness and I/sig (I) values displayed after data processing. First, check the Rmerge value. The value represents the degree of agreement of equivalent diffraction spots, so the smaller value, better data quality. Re-measurement is recommended when the Rmerge value is extremely large (Rmerge＞20%). It is also important to check the Rmerge in the highest resolution shell, which serves as a basis for determining from which resolution the data should be cut-off. Researchers can adopt their own criteria for the determination, e.g., adopting Rmerge of 60% or smaller as a cut-off for the highest resolution shell, or accepting Rmerge exceeding 100% in the highest resolution range when I/sig (I) is equal to or larger than a certain value.

The next value to be checked is completeness. The actual completeness values obtained after data processing often become low, which can be attributed to overestimation of mosaicity of the protein crystals or short distances between diffraction spots due to long lattice parameters. To avoid such problems, it should be effective to use smaller boxes for intensity integration during data processing.

Check the I/sig (I) values at individual resolutions. Extremely small values (I/sig (I)＜1.5) indicate that no diffraction spots are observed or their intensities are too weak, so the data from the corresponding resolutions should be excluded upon processing.

6. Conclusion

Regarding data collection and processing, innovative detector systems, high-intensity X-ray sources and newly developed software have drastically expanded the range of crystals that can be analyzed. Nevertheless, selection, pick-up and freezing steps of protein crystals involve a lot of intervention by the experimenters. The quality of data obtained from a protein crystal can be substantially improved by strictly performing these steps. Screening of protein crystals using “in situ” screening devices or adapters should enable efficient checking of crystal quality. Skills for mounting and freezing crystals can be improved by repeatedly performing these procedures, so it is desirable to have enough practice with lysozyme or thaumatin crystals. Optimization of freezing conditions requires steady efforts but will provide noticeable improvements, so it is recommended to patiently test several cryoprotectants.