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Perspective

A Short Review on Cryoprotectants for 3D Protein Structure Analysis

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Abstract: The three-dimensional structure of protein is determined by analyzing diffraction data collected using X-ray beams. However, X-ray beam can damage protein crystals during data collection, lowering the quality of the crystal data. A way to prevent such damage is by treating protein crystals with cryoprotectants. The cryoprotectant stabilizes the protein crystal and prevents lowering the quality of the diffraction data. Many kinds of cryoprotectants are commercially available, and various treatment methods have also been reported. However, incorrect selection or treatment of such cryoprotectants may lead to deterioration of crystal diffraction data when using X-ray beams.

Keywords: X-ray crystallography; protein crystals; cryoprotectant

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1. Introduction

The first protein crystal was observed about 180 years ago [1,2]. However, crystallizing protein remained questionable until the 1880s, when plant seed proteins were crystallized for purification [3,4]. Since then, the PDB (Protein Data Bank) has been established in 1971 [5], and more than 170,000 biological macromolecular structures have been registered in the PDB. About 85% of the biological macromolecule structures registered in the PDB were determined by X-rays [6].

X-ray crystallography is the most advantageous technique for determining protein structure [7]. It typically consists of nine steps: target protein selection, genetic engineering for increase the quantity of target proteins, purification, crystallization, treatment with cryoprotectants before freezing via liquid nitrogen, data collection, calculating the electron density map, refinement & validation, and finally the model building of target proteins [8] (Figure 1). The refined 3D structure of target protein gives new ideas to creating mutants of the target protein, i.e., the better functioning target protein in the cell.

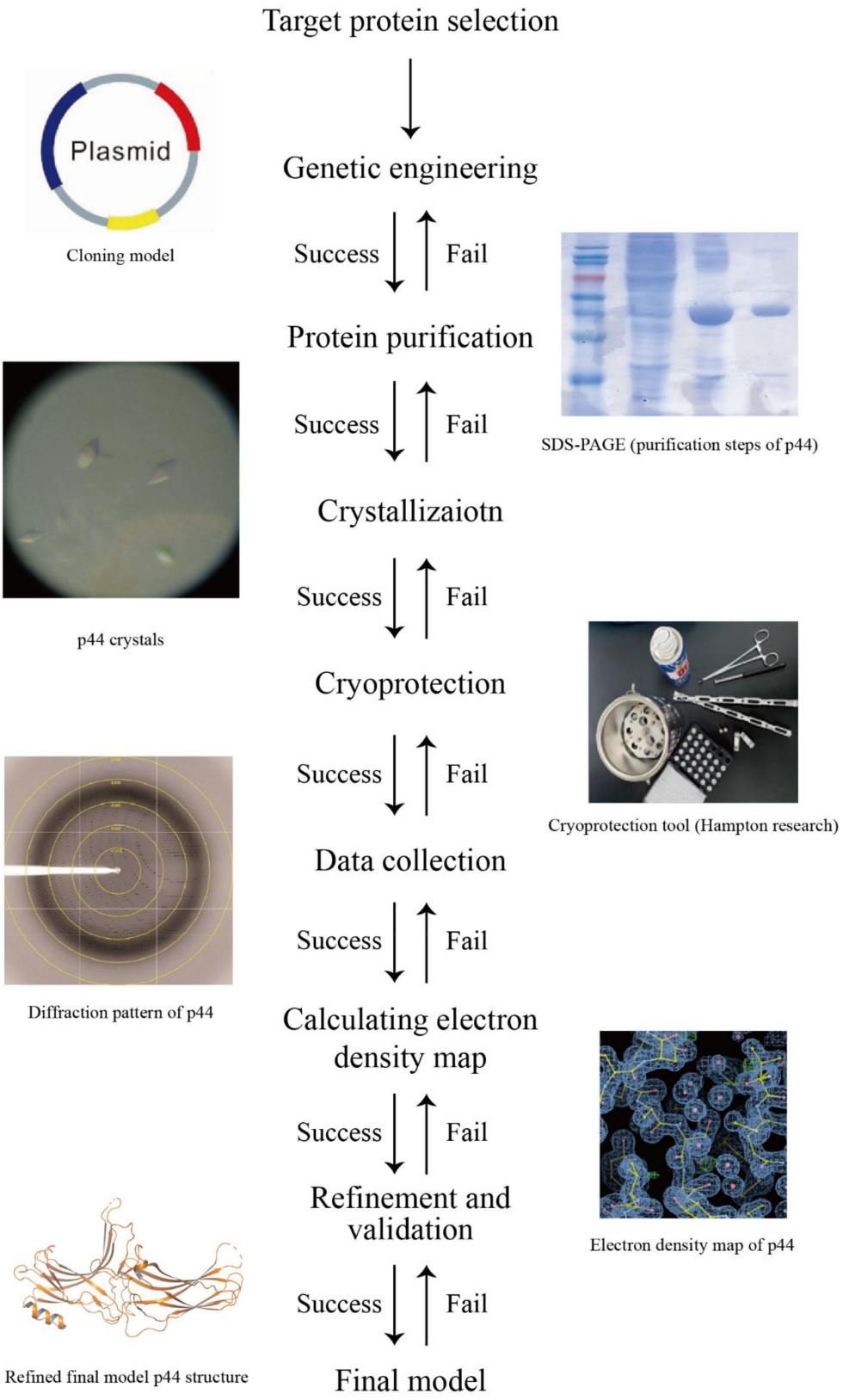

Figure 1. Overall processes of protein structure analysis using X-ray. p44 (PDB entry: 4J2Q [9]) also known as a splicing variant of arrestin that is actively working on in our laboratory, has been used for this figure, i.e.,SDS-PAGE, crystal, diffraction pattern, electron density map and final model).

Protein crystals kept at room temperature dramatically deteriorate when they are exposed to high-intensity X-rays [10]. Therefore, protecting and/or lowering radiation damage of protein crystals is essential for collecting better data sets. Sometimes it is possible for protein crystals exposed at 100 K to tolerate a much longer total X-ray exposure without seriously affecting the diffraction quality, so it can generally collect more than one complete dataset from a single crystal [11]. However, protein crystals also contain water, which transforms into hexagonal ice during cooling, expanding its volume [12]. Therefore, a cryoprotectant, such as sugars, glycerol, ethylene glycol, and polyethylene glycols (PEG) [13,14], that induces protein dehydration is essential (Figure 2) [15].

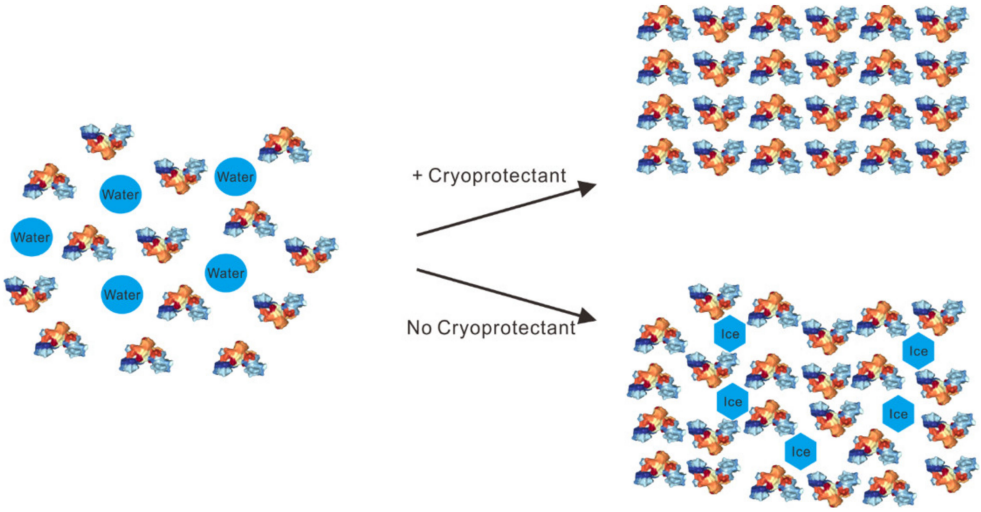

Figure 2. The basic principle of cryoprotection in protein crystals. Directly frozen and/or mis-selected cryoprotectant used protein crystals lead to ice rings on the diffraction caused by cooling environment. The reason is that water molecules contained in protein crystals undergo phase transition from water to hexagonal ice through rapid cooling with liquid nitrogen. This causes the volume of protein crystals to expand, destroys the crystal lattice, and leads to lowering the quality of data. Therefore, proper treatment with cryoprotectants in protein crystals is indispensable for better quality X-ray crystal data.

Caution should be exercised because high concentration cryoprotectants may also cause various side effects to protein crystals, such as volume reduction and morphology change of protein crystals, reductions of scattering contrasts, and an increase in solvent viscosity. However, the studies of cryoprotectants are few. This short review introduces widely used cryoprotectants, the cause and/or effect of cryoprotectants during cryoprotection, and their effectiveness on the diffraction quality of protein crystals.

2. What Is Cryoprotection, and When Do We Use Cryoprotectants?

Cryoprotectants inhibit and preserve protein crystal damage during freezing. Cryoprotection is the immersion of crystals in cryosolution for a short period, typically less than 10 s or longer than 1 day [16–18]. Some cryoprotectants may dissolve protein crystals; therefore, soaking times (damage) should be stringently monitored [19,20]. Cryoprotectant use also depends on crystallization conditions. Since the crystallization precipitant is almost identical to the cryoprotectant, the mother liquor is used for cryoprotection. If the precipitant concentration of the mother liquor is insufficient to protect the crystal, an additional cryoprotectant can be added.

Various types of cryoprotectants, such as salts, organic solvents, sugars, polyols, and polymers, are listed in Table 1. In many cases, the low molecular weight PEGs, such as PEG 200, 400, and 600, are appropriate cryoprotectants. Low molecular weight polyols (PEGs), sugars, and organic solvents penetrate the crystal lattice and cause dehydration [19,21,22]. Polymers, including high molecular weight PEGs, such as PEG 3350, 4000, and 6000, do

not penetrate crystals but coat the crystal lattice, and are referred to as non-penetrating cryoprotectants [23]. Similarly, oils do not penetrate crystals but provide barriers between the crystal surface and air. Alcohols and salts also act as dehydration molecules via vapor and osmotic effects.

Table 1. The types of cryoprotectants.

Penetrate				
Sugars (25~50%)				
Glucose	Sucrose	Maltose	Xylitol	Inositol
Trehalose	Raffinose	Erythriol		
Polyols (25~50%)				
Glycerol	Ethylene glycol	Diethylene glycol	Propylene glycerol	1,2-propanediol
2,3-butanediol	1,6-hexanediol	2-methyl-2,4-pentanediol		
Low weight PEGs (25~50%)				
PEG 200	PEG 400	PEG 600 etc		
Non-penetrate				
High weigh PEGs (25~50%)				
PEG 3350	PEG 6000	PEG 8000 etc.		
Oils				
Paraffin oil	Olive oil	Canola oil	Perfluoropolyether oil,	
Parabar 10312	Turbomolecular pump oil			
Others				
Vapor phase (alcohols)				
Methanol	Ethanol	Isopropanol		
Osmotics (salts, 50~90%)				
Lithium acetate	Lithium chloride	Lithium formate	Sodium malonate	Magnesium acetate
Sodium chloride	Sodium formate	Sodium nitrate	Ammonium sulfate	
				(concentration)

In PDB structures reported in approximately 1000 publications (2015–2021), polymers have been used as precipitating agents in 63% of structures (Figure S1a,b). Crystallization using organic compounds and salts as precipitants were used in 22% (Figure S1a,c) and 15% (Figure S1a,d) of structures, respectively. Since polymer- and organic compound-mediated crystallization has been performed at sufficient concentrations for cryo-cooling, such cryoprotectants are used less frequently when compared with salt crystallization approaches (63%, Figure S2a). The most frequently applied added cryoprotectant was penetrating cryoprotectants such as glycerol and/or EG (Figure S2b). This statistic result was similar to previous research result by Farley, C & Juers, DH [24]. However, cryoprotection using vapor and osmotic effects is rarely used (Figure S2b).

Thermal contractions are classified with contraction coefficients according to the solvent temperature. The thermal contraction value of cryoprotectants at cryogenic temperatures is important for determining the concentration of penetrating cryoprotectant for successful cryoprotection. Indeed, glycerol, as a popular cryoprotectant, is used at 25–30%, which is the concentration of glycerol to the equilibrium between extension and contraction values at 77 K [25]. Further, 20% DMSO is close to the equilibrium between extension and contraction values at 77 K; however, DMSO is limited due to its biochemical toxicity.

3. X-ray Damage and Diffraction Quality

The damage to protein crystals by X-rays occurs in stages. Nave [26] reported that radiation induces damage to crystals either directly or indirectly. In crystal damage, direct molecular destruction by radiation is called primary damage. Secondary damage refers

to the damage caused by free radicals produced by direct and/or indirect causes after radiation exposure. In addition, the crystal lattice may become unstable or destroyed due to primary and secondary damage. This phenomenon is called the domino phenomenon [27].

Various studies have reported the damage to protein crystals caused by X-rays [28–31]. Alkire et al. reported that various commercial cryoloops determine the differ diffraction data quality according to the stiffness and aperture diameter of the cryoloop [32]. Michael et al. developed crystals in cryoloops and collected diffraction data at 100 K in cryoprotection-free conditions. These authors suggested this method was advantageous as it could lead to technical advances, such as automated systems for protein structural analyses using X-rays, crystallization, cryoprotection, and data collection [33].

Although the cryoprotection condition is more limited than the crystallization condition (commercial crystallization screening kits: about 2500 and cryoprotection screening kits: about 100) (Table S1), it is evident that one of the important factors in preventing the damage and affecting the diffraction pattern is crystal cooling. If this is processed incorrectly, i.e., it causes ice rings within the diffraction pattern and damages the crystal itself. Crystals were grown using ammonium sulfate (2.8–3.2 M) causes ice rings and low data quality, despite being able to protect the crystals by the osmotic effect (Figure 3a,b). However, mis-cryoprotection can be improved using glycerol and various cryoprotectants (Figure 3c,d). Glycerol, which is popularly used for cryoprotection, affects carbonic anhydrase II activity. However, conformational changes in the carbonic anhydrase II structure are not identified in active and substrate binding sites, despite glycerol being used as a cryoprotectant [34].

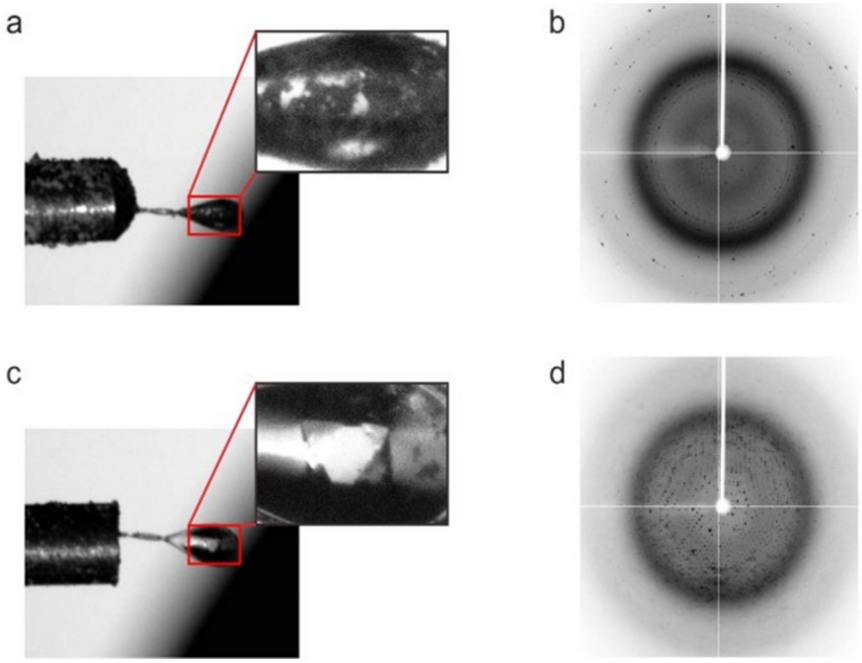

Figure 3. The importance of cryoprotection in diffraction quality. Incorrect cryoprotection of GAPDH protein forms an ice ring and lowering the quality of X-ray data (**a**,**b**). However, correct selection of cryoprotection for GAPDH crystal leads to much better quality of X-ray data (**c**,**d**).

4. How to Improve the Diffraction Quality of Protein Crystal?

Sometimes, high intensity of X-ray beam induces the poor quality of X-ray data and unsuitable for diffraction studies. These can be overcome by internal factors of synchrotron, such as attenuation time, oscillation range, and exposure time, but they cannot be a fundamental solution [35]. The post crystallization method can help to solve this problem. Post crystallization methods can convert poor diffracting crystals into data quality crystals through the various cryoprotection method [7].

Crystal annealing reduces the disorder of the flash cooling method and can increase the diffraction quality of the protein crystals [36]. There are three different types of crystal annealing: macromolecular crystal annealing (MCA) and flash annealing (FA). MCA removes a cryocooled crystal from the cold gas stream. It places in 300 mL cryo-solution, and after 3 min of equilibration, the crystal is re-cooled in cryo-stream [36]. The FA method blocks the cold-stream for 1.5–2 s three times and 6 s for the interval of each thawing step [37]. MCA treatment is better and more reproducible than others, the FA method is defective for most of the crystals [38].

Crystal dehydration is a treatment that produces the most remarkable improvements in the diffraction resolution of protein crystals [39]. Dehydration removes the excess solvent, tightens the protein molecules, and reduces the size of solvent channels. By this way, it improves the crystal order and diffraction resolution. Many studies have been reported that crystal dehydration method can convert poor quality crystals (>10 Å) to high-resolution data quality crystals (<2 Å) [40–45]. Removing excess solvent can make successful flash cooling easy, especially for large initial solvent contents crystals. After dehydration, protein crystals change the structural transformation and yield the alternative crystal packing; it is not possible or difficult to achieve directly during the crystal growth stage. Therefore, crystal dehydration is the most effective treatment for improving the crystal diffraction properties than the other post crystallization treatments [7].

Crystal soaking is similar to dehydration treatment. Dehydration can cause the shrinking of the crystal lattice and lower the solvent content of the crystal. Crystal soaking treatment without changing the unit cell or solvent content and notably improves the crystal diffraction quality. A higher concentration of precipitating agents can increase the quality of the diffraction resolution without evaporation or decrease the unit cell parameters. Glycerol is the most widely used cryoprotecting agent in crystal soaking solutions to improve the quality of protein crystals [46].

In the multistep soaking method, which is one of the alternative methods, crystals are soaked 2–3 times using different cryoprotectant solutions [47]. This method is considered useful when the diffraction pattern is not optimized by a commercially available cryoprotectant kit. When either of the two cryoprotectants has only a marginal effect on the protein crystal, the combining of the cryoprotectants (two or sometimes more) show a significant improvement in crystal quality. This method, however, may require as much time as that required by the previous method because it searches for the second and subsequent cryoprotectants.

The other approach is reported to increase the diffraction data quality in cryoprotection process. High-pressure cryocooling has been devised as an alternative method and has been successfully applied in various technical and scientific studies for cryoprotection of protein crystals [48–50]. This method requires the conservation of crystal hydration because pressure is applied to the crystal using dry helium gas. This method involves mounting the protein crystals from the native mother liquor onto a cryoloop using a droplet of oil, applying a pressure of 200 MPa in He gas to the crystals, cooling the crystal under pressure and then releasing the pressure. The crystal is then removed from the apparatus under liquid nitrogen and handled thereafter in a manner similar to a normal cryocooled crystal. These methods are not sufficiently adequate for low resolution diffracting crystals since the relatively high background scattering due to the hydrating materials.

5. The Problem in Structural Analysis

The chemical properties of cryoprotectants can absorb radicals generated by radiation. It has also been reported to reduce damage to crystals, primarily due to changes in protein-protein interactions [51]. However, added molecules, such as cryoprotectants, should not affect the protein structure except for the ligand. It rarely occurs using the penetrate cryoprotectant.

Kim showed that trehalose induces morphological changes in the structure of GAPDH through binding to S-loop [52]. Trehalose is one of the cryoprotectants that induces in-

hibiting protein hydration [53]. The apo- and holo-structures of GAPDH rotate 2.4° and 3.1°, respectively, compared to the trehalose bonded structure. There are two hypotheses. One is that the change in shape is a result of the penetration of the cryoprotectant. Some penetrating cryoprotectants, such as low-weight polymers, polyols, and saccharide groups, bind to the protein structure. The GAPDH structure in the PDB has been reported to bind to GAPDH as a cryoprotectant. However, the cryoprotectant does not affect key domains such as the active site and protein interaction domain. Interestingly, trehalose binds to the S-loop of GAPDH, a key residue in the protein-protein interaction [54,55]. In contrast, other cryoprotectants such as maltose and sucrose do not bind GAPDH in Kim's work. It is supposed that the protein structure might undergo a conformational change in some cases through cryoprotection because of hydration and dehydration in internal cavities as well as protein surface [56]. In these reasons, the collection of X-ray diffraction has been conducted at room temperature (RT), recently.

X-ray diffraction data of dihydrofolate reductase (DHFR) were collected at both RT and cryogenic temperatures (CT). As expected, CT data set resolution was higher than the RT data set. However, while more ensemble water molecules were generated at RT, interestingly, the number of water molecules interacting with proteins was greater at CT. In addition, Arg 52 residues interacting with folate were more flexible at RT [57]. There were also reported that the hidden ligands binding site neither active nor catalytic site binding site of some protein could be revealed in RT conditions, but not in CT conditions [58,59]. Previous studies also reported that crystallography conducted using serial femtoseconds and microfluidic platforms were useful during RT conditions, but required a large amount of protein crystals for measurement [60]. In addition, the crystal damage of X-ray beam is still unsolved. Therefore, it is necessary to find suitable cryoprotectants and methods when proceeding with crystal cooling [61–64].

6. Conclusions

Cryocrystallographic methods are essential in protein crystallography. In X-ray crystallography, cryoprotection plays an important role in improving data quality. Recently, cryoprotection has been tried under various conditions, such as the multistep soaking method and high pressure, to improve data quality. However, cryoprotectants can also affect the structure of the protein. Due to various factors, the choice of cryoprotectant depends on practical experience. Therefore, this short review could have a positive effect as an empirical basis for the future in X-ray crystallography.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cryst12020138/s1, Figure S1. The statistic analysis of precipitants for crystallization in the structures reported to the PDB in the period of 2015 to 2021, Figure S2. The use of additional cryoproteiction, Table S1. Lists of commercially available cryoprotectants from two representative companies

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