Chapter 8

Screening of Cryoprotectants and the Multistep Soaking Method

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Abstract

Crystals obtained from an initial crystallization screening are not always of sufficient quality for structural determination at atomic resolution. For this reason, post-crystallization treatments such as cryoprotection and dehydration have frequently been utilized to improve the crystal quality. In addition, several recent studies have shown that cryoprotectants can interact with the proteins in the obtained crystal and further stabilize them, leading to further improvement of the crystal quality. In this chapter, we propose a multistep soaking method in which crystals are sequentially soaked in two to three cryoprotectant solutions. This method was found to be effective for improving the crystal quality. However, since the screening of cryoprotectants for use in this method involves much trial and error, it is important to record each step of the screening in a systematic manner.

Keywords Cryoprotectants, Post-crystallization treatment, Artificial mother liquor

1 Introduction

1.1 Post-crystallization Treatment

While X-ray crystallography is a powerful tool to determine the tertiary structure of biological macromolecules at atomic resolution, it generally requires high-quality crystals that diffract to better than 3 Å resolution. Therefore, numerous attempts have been made to establish a method for obtaining crystals of sufficient quality. In particular, the development of crystallization screening kits and crystallization robots has dramatically increased the success rate and efficiency of the initial crystallization screening of biological macromolecules [1–5]. Nonetheless, the obtained crystals are not always of sufficient quality for crystal structure determination at atomic resolution. Thus a reliable method for improving the quality of the crystals is needed.

One of the frequently utilized methods to improve the crystal quality is changing the target protein to its homologue. Crystallization of a homologue of the target protein sometimes yields a high-quality crystal [6–10]. Deletion of intrinsically disordered

Toshiya Senda and Katsumi Maenaka (eds.), *Advanced Methods in Structural Biology*, Springer Protocols Handbooks,
DOI 10.1007/978-4-431-56030-2_8, © Springer Japan 2016

region(s) from the target protein is also effective for improving the crystal quality [11]. However, in some cases, we cannot adopt these strategies. When our target protein is not a recombinant protein, for example, preparation of a deletion mutant is impossible. If homologues of the target protein cannot yield crystals better than the original one, we need to use the original crystal. In these cases, another strategy for crystal quality improvement is required. Post-crystallization treatment [12, 13] can be applied even in these difficult cases. Post-crystallization treatment improves the crystal quality by subjecting the crystal to various combinations and levels of soaking, freezing, and humidity [14–21].

1.2 Cryo-crystallography

In recent years, most diffraction data have been collected at cryogenic temperature to avoid radiation damage by the synchrotron X-ray radiation [22, 23]. Crystals are flash-cooled before data collection and kept frozen in a cold N_2 flow, typically at 100 K, during the diffraction data collection. The techniques of diffraction data collection at cryogenic temperature were developed in the early 1990s, when the use of synchrotron radiation became common in the field of protein crystallography [23, 24]. Protein crystals typically consist of approximately 50 % water molecules by volume [25], and the water molecules surrounding and/or inside the protein crystal form crystalline ice upon freezing, damaging the protein crystal. Furthermore, the crystalline ice causes strong circular diffractions known as ice rings at around 3.7 Å resolution, which hampers the collection of high-quality data from the frozen crystal (Fig. 1a) [23]. For this reason, the water molecules should be frozen in an amorphous state to avoid crystalline ice formation (Fig. 1b). In the

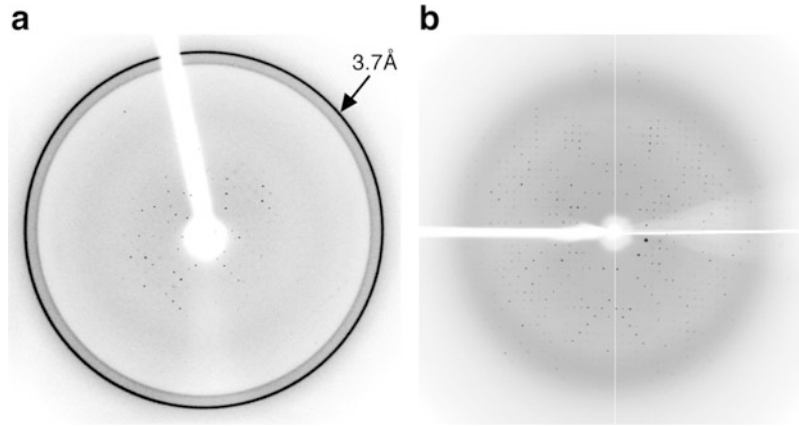

Fig. 1 A diffraction pattern with ice rings. (**a**) A TAF-IβΔC crystal was flash-cooled without cryoprotectant solution. Ice rings were observed at around 3.7 Å resolution. (**b**) A TAF-IβΔC mutant (Leu104Met/Leu145Met/Leu166Met) crystal was flash-cooled using artificial mother liquor containing 30 %(w/v) trehalose. No ice rings were observed because the level of cryoprotection was appropriate

1960s, it was discovered that small compounds such as sucrose could be utilized to freeze water molecules in an amorphous state [26]. In 1975, the replacement of crystallization solution with an organic compound was reported to be an effective method for crystal freezing [27]. The method of crystal freezing was rapidly improved in the 1990s, when protein crystallographers began to utilize many organic compounds and sugars as cryoprotectants [23, 24]. Today, kits for screening the cryoprotectants to be used in crystal freezing are commercially available.

1.3 Cryo-conditions

While cryoprotectants have been widely utilized in crystal freezing, most crystallographic investigations have been carried out without intensive screening of the cryoprotectants. Since inappropriate selection of a cryoprotectant leads to poor diffractions, optimization of the cryo-conditions is critical to obtaining high-quality diffraction data. Interestingly, several analyses have revealed that cryoprotectants occasionally interact with protein molecules in the crystal, resulting in the improvement of the crystal quality by stabilizing the target protein [28–33]. Therefore, cryoprotectants can also be used to improve the crystal quality by stabilizing proteins in the crystal. In this chapter, we describe methods to improve the crystal quality using cryoprotectants. We begin by explaining the basic soaking technique. We then describe the methods used to screen for optimal cryo-conditions and the protocol of the multistep soaking.

1.4 Basics of Soaking Experiments

Since the cryoprotection of the protein crystal is achieved by soaking, it is essential to prepare artificial mother liquor (or standard buffer) before the soaking experiment. Otherwise, the crystals will be damaged during soaking and the results will be poor. It has been established that artificial mother liquor can maintain the crystal of a target protein for at least 2–3 days without damaging it [34]. An artificial mother liquor can be prepared based on the conditions of the crystallization solution (reservoir solution). There are two critical conditions for the artificial mother liquor. First, the pH of the artificial mother liquor should be adjusted to that of the droplet solution that produces the crystal. The difference of pH should be less than 0.1 in order to avoid pH shock when transferring a crystal from a crystallization droplet to the artificial mother liquor. Second, the concentration of the precipitant(s) should be optimized; the concentration needs to be increased by 10–20 % from that of the crystallization solution to avoid dissolution of crystals. Once the conditions of the artificial mother liquor are fixed, the artificial mother liquor can be utilized for a variety of soaking experiments. The artificial mother liquor, of course, can be utilized for typical soaking experiments to prepare crystals of the protein-small compound complexes.

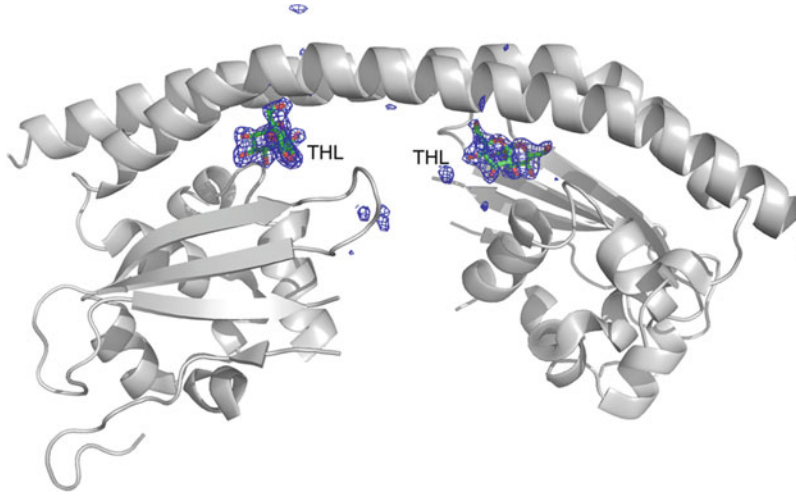

Fig. 2 Trehalose molecules bound to TAF-Iβ. Trehalose (*THL*) molecules can be seen located between the two domains. The mFo-DFc densities are contoured at about 3.0 σ

After fixing the conditions of the artificial mother liquor, we can start the screening of cryoprotectants. It is convenient to use commercially available screening kits for the initial screening. This first screening of cryoprotectants should be done with 20–30 regents. The effects of the cryoprotectant will differ under different soaking times and/or soaking temperatures, and these parameters are generally optimized after the initial screening.

As described above, some cryoprotectants interact with the target protein in the crystal. This type of interaction has been suggested to stabilize the target protein in the crystal and improve the crystal quality [28–33]. In the case of TAF-Iβ, which is a histone chaperone that interacts preferentially with the histone H3-H4 complex, molecules of trehalose, which was used as a cryoprotectant, were found in the crystal structure [31, 32]. These trehalose molecules were located between two domains and interacted with these domains by forming intensive hydrogen bonds. These interactions seemed to stabilize the protein structure and improve the crystal quality (Fig. 2). Since most of cryoprotectants have several polar groups, such as hydroxyl and carbonyl groups, cryoprotectants are likely to interact with protein molecules via hydrogen bonds. It is therefore reasonable to utilize more than one cryoprotectant to stabilize the protein structure. Indeed, a combination of several cryoprotectants has been proven effective for crystal quality improvement [35].

1.5 Crystal Annealing

One of the common problems in crystal freezing is the increase of crystal mosaicity [24]. This seems to happen even when the water molecules are frozen in an amorphous state. The high mosaicity can sometimes be improved by a crystal annealing procedure, in which

a frozen crystal is kept at room temperature to thaw it and then refrozen by a cold N_2 flow after several seconds. Since this annealing procedure sometimes dramatically improves the crystal mosaicity [16, 17], crystal annealing has frequently been applied in conjunction with cryoprotection.

1.6 Evaluation of Diffraction Images

In the screening of the cryoprotectants, particularly for the multistep soaking method, we need to examine numerous diffraction images to select suitable soaking conditions. It is critical that the diffraction images should be evaluated using the same criteria. In this manuscript, we utilize two measures for crystal resolution, the *maximum resolution* and *resolution limit*. The maximum resolution is defined on the basis of the statistics of diffraction data processing/scaling. We define the maximum resolution as the resolution that satisfies $R\text{merge} < 0.5$ and $I/\sigma(I) > 3$ at the outermost resolution shell. The second measure is the resolution limit, which is determined by the visual inspection of diffraction images. Due to the lack of numerical criteria on the maximum resolution, the resolution limit may show some degree of deviation. Our analysis, however, revealed that these two values show a correlation if the resolution limit is determined carefully (Fig. 3). In the screening process, we need to evaluate the quality of each crystal with a few snapshot images. Since we use only a limited number of crystals for the full data collection, it is not practical to compare the crystal quality using the maximum resolution. We therefore need to utilize the resolution limit of the crystal to compare the crystal quality. It is of course possible to use other criteria; some programs have been developed to estimate the resolution of the crystal from a diffraction image. The most important point is, however, that the criteria should be the same throughout the screening process.

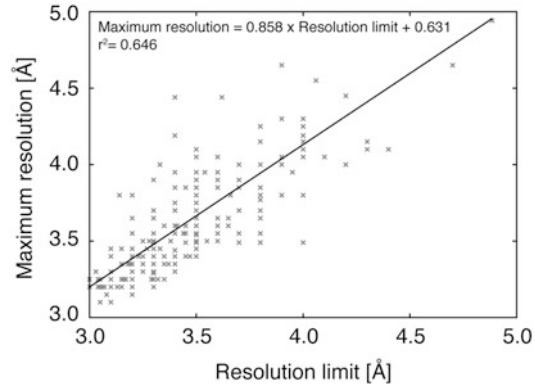

Fig. 3 Correlation between the maximum resolution and the resolution limit. The resolution limit showed reasonable correlation with the maximum resolution. The resolution limit, which is determined by visual inspection of a few diffraction images, could be used in the cryoprotectant screening

2 Materials

2.1 Chemicals

Sugars, alcohols, PEGs, and organic compounds (glycerol; ethylene glycol; PEG200; PEG400; PEG600; PEG4000; polyvinylpyrrolidone K 15, (+/−)-2-methyl-2,4-pentanediol (MPD); 1,6-hexanediol; 1,2-propanediol; dimethyl sulfoxide (DMSO); 2-propanol; ethanol; methanol; D-(+)-sucrose; meso-erythritol; xylitol; D-(+)-raffinose; D-(+)-trehalose dihydrate (THL); D-(+)-glucose, etc.) have frequently been utilized as cryoprotectants. Screening kits of cryoprotectants are commercially available from several companies. Special care must be taken when using PEG as a cryoprotectant. While PEG is available from various suppliers, the quality of PEG will differ widely among these sources. When obtaining PEG, therefore, check the pH of the PEG solution; again, the pH values of the PEG solutions will differ among vendors.

2.2 Glassware

For the soaking experiments, a depression glass is useful (Fig. 4). The glass should be siliconized before use. A cryoloop is required to transfer crystals between the solutions. A stereomicroscope is utilized for observation of the soaked crystals. It is convenient to use the stereomicroscope in a cold room, because soaking at 4 °C is frequently effective.

Fig. 4 Depression glass. Two droplets are placed at the bottom of the well. The well should be sealed with a cover glass using grease

3 Methods

3.1 Preparation of Artificial Mother Liquor

1. Measure the pH of the crystallization solution that harbors crystals of your target protein.
2. Prepare some candidates of the artificial mother liquor on the basis of the crystallization (reservoir) solution (Table 1). Their pH should be adjusted to that of the crystallization

Table 1
Examples of artificial mother liquor and cryoprotectant solution [31, 32, 35–39]

Protein name	Crystallization conditions	Artificial mother liquor	Cryoprotectant solution
BphA4	1.7–2.1 M sodium formate	2.5 M sodium formate	27.5 %(v/v) glycerol
	100 mM sodium acetate pH 5.3–5.4	0.1 M sodium acetate pH 5.4	2.5 M sodium formate
			0.1 M sodium acetate pH 5.4
CagA(1–876)	7–10 %(v/v) ethanol	20 %(v/v) ethanol	28 %(w/v) trehalose
	50 mM Tris-HCl pH 7.0–7.1	50 mM Tris-HCl pH 7.0	20 %(v/v) ethanol
			50 mM Tris-HCl pH 7.0
DDO (D-aspartate oxidase)	5–10 %(w/v) PEG8000	20 %(w/v) PEG8000	20 %(v/v) glycerol
	100 mM potassium dihydrogen phosphate	100 mM potassium dihydrogen phosphate	20 %(w/v) PEG8000
	100 mM sodium acetate pH 4.7	100 mM sodium acetate pH 4.7	100 mM potassium dihydrogen phosphate
			100 mM sodium acetate pH 4.7
DSD (D-serine dehydratase)	12–15 %(w/v) PEG4000	30 %(w/v) PEG4000	30 %(v/v) glycerol
	10 %(v/v) 2-propanol	10 %(v/v) 2-propanol	20 %(w/v) PEG4000
	100 mM MES pH 6.5	100 mM MES pH 6.5	10 %(v/v) 2-propanol
			100 mM MES pH 6.5
TAF-Iβ	2.4–2.85 M ammonium sulfate	2.75 M ammonium sulfate	30 %(w/v) trehalose
	200 mM potassium sodium tartrate	200 mM K/Na tartrate	2.75 M ammonium sulfate
	30 mM magnesium chloride	30 mM magnesium chloride	200 mM K/Na tartrate
	100 mM sodium citrate pH 5.4–5.5	100 mM sodium citrate pH 5.4	30 mM magnesium chloride
			100 mM sodium citrate pH 5.4

solution (measured in step 1). The concentration of precipitant (s) should be increased by 10–20 %.

3. Soak crystals in each of the prepared solutions. Crystals can be transferred from a crystallization droplet to the prepared solution by a cryoloop. Do not damage the crystal when transferring it. In particular, avoid touching the crystal with the cryoloop. A depression glass (Fig. 4) is useful for the soaking experiment. After the crystal transfer, the well of the depression glass should be sealed to avoid evaporation of the artificial mother liquor. A typical volume of the artificial mother liquor for the soaking experiment is 5–20 μL.
4. Observe the soaked crystals with a stereomicroscope. Check for cracks on the crystal surface just after soaking. If the crystals crack immediately after soaking, the concentration of the precipitant should be changed. Also, it is useful to check the pH of the solution. Finally, sometimes low-temperature soaking (e.g., soaking at 4 °C) may prevent the crystal damage.
5. After the observation, the depression glass should be stored in an incubator. The temperature will usually be the same as that of the crystallization.
6. Crystals should be observed each day, with careful monitoring for cracks on the surfaces and change in the crystal size due to dissolution. The goal is to identify conditions that do not crack and dissolve the soaked crystals. The best means of accomplishing this is to take a photo of the soaked crystals each day in order to monitor their status.
7. If possible, it is better to check diffractions from a crystal soaked in the artificial mother liquor.
8. When you cannot stop the dissolution of the crystal in the artificial mother liquor, try to add your target protein in the artificial mother liquor; the concentration of the protein is usually less than that of the crystallization conditions.
9. Table 1 shows examples of the artificial mother liquor. Compare the conditions of the crystallization solution and artificial mother liquor.

3.2 Screening of Cryoprotectants

1. Prepare a cryoprotectant solution on the basis of the conditions of the artificial mother liquor. The concentration of a protectant is approximately 15–30 % (w/v, v/v). It is convenient to prepare a 2× artificial mother liquor and 30–60 % solution of a

cryoprotectant and mix them in a 1:1 ratio. After mixing the solutions, the pH of the mixture should be adjusted to that of the artificial mother liquor.

2. Several crystals should be soaked in one cryoprotectant solution to check for reproducibility. It is highly recommended that the size of each crystal be recorded. Just after soaking, check the appearance of the crystal. Here again, for this purpose, it is best to take a photo of the soaked crystals. In some cases, the crystals will be damaged immediately after soaking in the cryoprotectant solution.
3. A soaking time of 30 s to 3 min is recommended for the initial screening.
4. After the soaking, the crystal is mounted on a cryoloop and frozen. Please note that freezing in a cold N_2 flow (*ca.* 100 K) and freezing with liquid nitrogen may result in different quality of crystal diffractions. Try both methods of freezing.
5. Take diffraction patterns (snapshots) from several directions ($\varphi = 0°, 45°, 90°$, etc.) and analyze the crystal quality. Check the resolution (resolution limit), mosaicity, shape of diffraction spots, and anisotropy carefully. Handling of the crystal frequently damages the crystal quality. To avoid this type of artifact, it is important to check the crystal quality with two to three crystals. While it is possible to statistically judge the significance of the difference in resolution, it is better to detect obvious differences in the crystal quality at the initial stage of the screening.
6. Try 20–30 cryoprotectants and select cryoprotectant solutions that give high-resolution diffractions.
7. Optimize the soaking time and temperature of the selected cryoprotectant solution(s).
8. When the crystals are damaged by soaking, try soaking at a low temperature (e.g., 4 °C). Sometimes low-temperature soaking dramatically improves the situation.

3.3 Multistep Soaking Method

1. Prepare the artificial mother liquor as described in Sect. 3.1.
2. Perform cryoprotectant screening as described in Sect. 3.2. Make a list of cryoprotectants that improve the crystal quality. Even if the effect of a cryoprotectant is marginal, it should be included in the list of the cryoprotectants that will be used in the multistep soaking. Even if the effect of a given

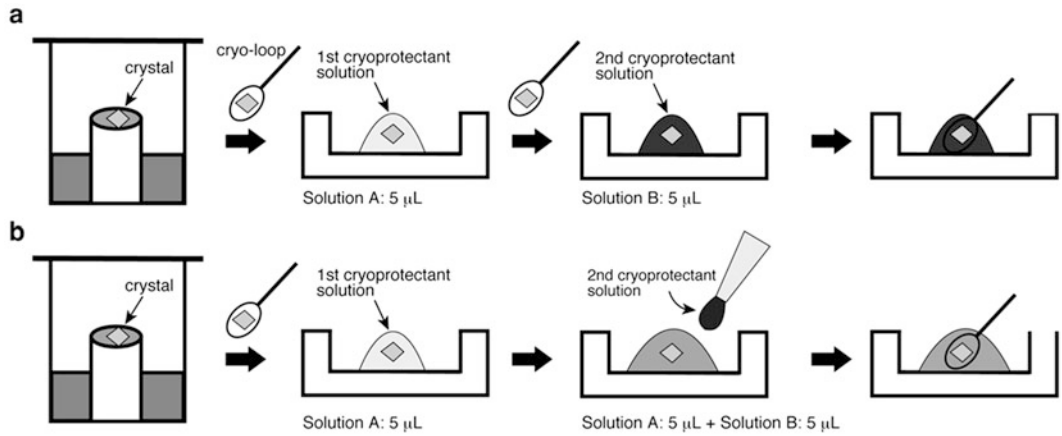

Fig. 5 Two methods for multistep soaking. (**a**) In the first method, a crystal is transferred from a crystallization droplet to the first cryoprotectant solution and then transferred to the second cryoprotectant solution. (**b**) In the second method, the crystal is soaked in the first cryoprotectant solution, and then a second cryoprotectant solution is added to the first

cryoprotectant is small, in combination with other cryoprotectants, it may dramatically improve the crystal quality.

3. Try the combinations of cryoprotectants listed above. When combining cryoprotectants, the order of soaking affects the results. There are two methods for the multistep soaking. In the first method, a crystal soaked in the first cryoprotectant solution is transferred into another cryoprotectant solution using a cryoloop (Fig. 5a). In the second method, the second cryoprotectant solution is added to the first cryoprotectant solution containing a crystal (Fig. 5b). A combination of two cryoprotectants is most typically used for the multistep soaking.
4. Examine the quality of the diffraction images. As described above, take the diffraction patterns from several different directions ($\varphi = 0°$, 45°, 90°, etc.) and analyze the crystal quality. Handling of the crystal frequently damages the crystal quality. To avoid this type of artifact, it is important to check the crystal quality with two to three crystals.
5. When you find a good combination of cryoprotectants, optimize the soaking time and temperature of each soaking.

When using the multistep soaking method, it is highly recommended that a spreadsheet program such as MS Excel be used to prepare a table (Table 2). This type of table is useful to systematically compare the quality of the crystals treated under various conditions.

Table 2
Data sheet for screening of cryoprotectant solution

				First step soaking		Second step soaking		Resolution	
ID	Crystal ID	Plate ID	Size [mm]	Cryoprotectant 1	Time	Cryoprotectant 2	Time	Max [Å]	Limit [Å]
###	Protein-yymmdd-###	###	##	##% ### ##% ## ## mM ## pH ##	## min	##% ### ##% ## ## mM ## pH ##	## min	###	###
001	Caga-091218-006	001	0.2	30 % trehalose 20 % ethanol 50 mM Tris-HCl pH 8.8	3 min	–	–	4.7	4.65
002	Caga-100528-001	058	0.5	17.5 % erythritol 40 % ethanol 50 mM Tris-HCl pH 9.0	0.3 min	–	–	3.4	3.70
003	Caga-100627-015	117	0.4	30 % trehalose 20 % ethanol 50 mM Tris-HCl pH 8.8	14 h	17.5 % erythritol 40 % ethanol 50 mM Tris-HCl pH 9.0	2.3 min	3.3	3.49
004	Caga-120128-034	471	0.3	30 % trehalose 20 % ethanol 50 mM Tris-HCl pH 7.0	30 min	30 % PEG1000 20 % ethanol 50 mM Tris-HCl pH 7.0	1.0 min	3.4	3.30

Acknowledgments

This study was supported by the New Energy and Industrial Technology Development Organization (NEDO), CREST (JST), Grants-in-Aid for Scientific Research on an Innovative Area (MEXT), and the Platform for Drug Discovery, Informatics, and Structural Life Science (MEXT) of Japan.

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