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Cryosalts: suppression of ice formation in macromolecular crystallography

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Quality data collection for macromolecular cryocrystallography requires suppressing the formation of crystalline or microcrystalline ice that may result from flash-freezing crystals. Described here is the use of lithium formate, lithium chloride and other highly soluble salts for forming ice-ring-free aqueous glasses upon cooling from ambient temperature to 100 K. These cryosalts are a new class of cryoprotectants that are shown to be effective with a variety of commonly used crystallization solutions and with proteins crystallized under different conditions. The influence of cryosalts on crystal mosaicity and diffraction resolution is comparable with or superior to traditional organic cryoprotectants.

1. Introduction

X-ray data collection at cryogenic temperatures has become an essential technique for crystallographic structure determinations of biological macromolecules (Hope, 1990; Watenpaugh, 1991; Rogers, 1994, 1997; Garman & Schneider, 1997; Garman, 1999). Freezing the crystals reduces radiation-induced decay so that one or more complete data sets can be taken from a single crystal. Such stability is useful in all studies, but it is especially important in multiple-wavelength anomalous dispersion (MAD; Hendrickson, 1991) for which multiple isomorphous data sets must be collected from the same crystal. Flash-frozen crystals may also diffract to higher resolution because of the absence of attenuation and scattering by the quartz or glass capillaries used in traditional crystal mounting. The absence of the capillary also allows smaller crystals to be used. In general, crystallographers agree that flash-freezing also eases physical manipulations of the crystals and lessens the probability of damage to or destruction of the sample.

Flash-freezing proteins for data collection requires mounting the crystal on or within a small loop of non-diffracting material. The crystal and loop are then plunged into a cryogen (such as liquid nitrogen or propane) or placed directly into the flowpath of a cryogenic gas flowing at the mounting site within the data-collection system (Teng, 1990). The crystals are maintained at approximately 100 K while collecting the data. For optimal results, the formation of crystalline or microcrystalline ice in the crystal and the solution holding the crystal should be suppressed. In a few cases, the crystallization solution and crystals freeze without ice formation. However, suppressing ice formation typically requires adding cryoprotectants (*e.g.* various polyalcohols) to the solution. Alternatively, various oils are used to coat a crystal either during or after blotting off the mother liquor.

For recent reviews, see Garman & Schneider (1997) and Rogers (1997).

The first attempts to use alcohols and glycols to reduce ice formation in macromolecular crystallography were reported by Low *et al.* (1966) in efforts to reduce radiation damage to orthorhombic insulin crystals. Later, the first successful uses of cryoprotectants involved soaking glutaraldehyde crosslinked lysozyme crystals in 50% glycerol (Haas, 1968) and 3.0 *M* sucrose (Haas & Rossmann, 1970). In the intervening years between these early studies and the present, a growing list of cryoprotectants such as glycerol, ethylene glycol, low molecular weight polyethylene glycols (PEGs), xylitol, erythritol and 2-methyl-2,4-pentanediol (MPD) have been used. The selection of traditional cryoprotectants, primarily sugars and alcohols, were based on the successful use of tissue-preservation agents by cryobiologists (*e.g.* Echlin, 1992).

Avoiding ice formation to obtain clear glasses is also a concern of spectroscopists. For much of the previous century, multimolar concentrations of ionic salts were used to engender glass formation in aqueous solutions. For that purpose, the most commonly used salt is LiCl, although any salt that is soluble enough and does not precipitate upon freezing can also be used. Examples of such salts and their solubilities are shown in Table 1. Interestingly, the use of such solutions with properties suitable for cryocrystallographic studies has never been reported.

The use of lithium formate, lithium chloride and other highly soluble salts to form ice-ring-free glasses suitable for macromolecular cryocrystallography are described here for the first time. These salts added to crystal stabilization solutions are effective cryoprotectants with properties comparable with or superior to the traditional organic compounds that are in common use.

Table 1
Solubilities of representative cryosalts in water.

Data from Söhnel & Novotny (1985). Lithium salts tend to form precipitates with phosphate.

Salt	Formula weight	Temperature (K)	Weight %	Molarity	Molar ratio† H_2O:salt
LiOAc	102.02	291	30.00	5.09	8.55
LiCl	42.40	273	76.90	18.14	3.44
$LiCHO_2$	69.97	291	27.85	3.98	7.80
$LiNO_3$	68.95	273	89.80	13.02	7.17
Li_2SO_4	90.39	273	80.00	8.85	17.12‡
$Mg(OAc)_2$	142.39	288	50.00	4.22	7.91‡
NaCl	58.44	273	26.27	5.43	9.11
$NaCHO_2$	68.01	273	30.84	5.50	8.47
$NaNO_3$	84.99	273	42.22	6.71	6.46

† Calculated from the molarity of water derived from experimental solution densities. ‡ Indicates three ions per mole. The rest have two.

2. Materials and methods

2.1. Materials

Crystallization reagents including the lithium, sodium and magnesium salts were reagent grade. Crystal Screen Kit I was purchased from Hampton Research.[1] Ribonuclease S and lysozyme were purchased from Sigma Inc.

2.2. Crystal growth

Crystals of ribonuclease S were grown at room temperature using the hanging-drop vapor-diffusion method. The well and droplet volumes were 1.0 ml and 10 µl, respectively. The final droplets of the ribonuclease S experiments contained 30 mg ml^{-1} RNase S, 0.1 *M* sodium acetate buffer pH 5.0, 60% saturated ammonium sulfate and 25% saturated sodium chloride (Gilliland *et al.*, 1994). Crystals of lysozyme were grown using a batch procedure from solutions containing 100 mg ml^{-1} lysozyme, 6.5%(*w*/*v*) sodium chloride and 0.1 *M* sodium acetate at pH 4.8.

2.3. Cryosalt additions to a fast screen

The cryo-test solutions for the Hampton Screen Kit I (Hampton Research, Laguna Beach, CA, USA) were prepared by mixing 20 µl of the Crystal Screen Kit I reagent with appropriate volumes of saturated solutions of cryosalts. A cryoloop was then filled with the liquid by passing it through the mixture in preparation for flash-freezing and diffraction experiments.

2.4. X-ray diffraction experiments

Equal volumes (20 µl) of a saturated lithium formate cryosalt solution and the crystallization mother liquor were mixed on a cover slip. The crystal was then transferred with a loop from the crystal-growth solution and either placed in or pulled through the cryosalt–mother liquor solution mixture. It was then transported into the cryogenic gas stream and mounted on the goniometer. The time from initial contact with the cryosolution until being frozen was in the range 15–20 s.

X-ray data was collected using a Brüker AXS, Inc. (Madison, WI, USA) electronic area-detection system and rotating-anode X-ray generator. During data collection, the crystals and crystal-mounting loops containing the solutions were maintained near 100 K in a nitrogen coldstream by an Oxford Cryosystems Cryostream (Oxford Cryosystems, UK). Diffraction data were processed with *X-GEN* (Molecular Simulations).

3. Results and discussion

3.1. Cryosalts: a new class of cryoprotectant

X-ray diffraction studies have established that frozen aqueous ionic solutions can produce glasses. Nevertheless, the mechanisms of glass formation are still being debated. In

[1] Certain commercial materials, instruments and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments or equipment identified are necessarily the best available for the purpose.

general, by lowering the freezing point close enough to the glass transition, crystal nucleation is suppressed because of sluggish transport (Dupuy *et al.*, 1982). However, in certain cases glass formation appears to be an equilibrium property rather than kinetically controlled (Sridhar & Taborek, 1988).

In practice, a binary salt alone in water generates a glass upon freezing when the ratio of molarities of water to salt are in the range 6–11 or less, depending on the ions' identities (Yamanaka *et al.*, 1993; Kanno, 1987). In essence, water molecules involved in ion hydration are so numerous that forming the continuous network of ice is difficult. For example, as Table 1 shows, saturated lithium formate has a water:salt molar ratio of about 8, while that of lithium chloride lies between 3 and 4. Both freeze as glasses when crystal-mounting loops are cooled from room temperature with a 100 K nitrogen-gas stream. In addition to the salts listed in Table 1, preliminary studies indicate that numerous alkyl-ammonium salts also provide the same protection. Complete results on the latter group will be reported in a subsequent publication.

Solution number, salt concentration	Buffer 0.1 *M*	Other components	No Salt	50%[a] $LiCHO_2$	50%[a] LiCl	50%[a] Li_2SO_4	%[b] $LiCHO_2$
1. 0.02 *M* Ca chloride	NaAc pH 4.6	30% (*v*/*v*) MPD	c	c	c	p	0
2. None	None	0.4 *M* K,Na tartrate	r	c	c	c	30
3. None	None	0.4 *M* NH_4 phosphate	r	r	c	r	80
4. None	Tris–HCl pH 8.5	2.0 *M* NH_4 sulfate	r	r	p	r	75
5. 0.2 *M* Na citrate	Na HEPES pH 7.5	30%(*v*/*v*) MPD	c	c	p	c	0
6. 0.2 *M* Mg chloride	Tris–HCl pH 8.5	30%(*w*/*v*) PEG 4000	r	c	c	c	35
7. None	Na cacodylate pH 6.5	1.4 *M* Na acetate	r	r	c	r	60
8. 0.2 *M* Na citrate	Na cacodylate pH 6.5	30%(*v*/*v*) 2-propanol	r	d	c	r	70
9. 0.2 *M* NH_4 acetate	Na citrate pH 5.6	30%(*w*/*v*) PEG 4000	r	c	c	p	10
10. 0.2 *M* NH_4 acetate	Na acetate pH 4.6	30%(*w*/*v*) PEG 4000	r	d	c	p	70
11. None	Na citrate pH 5.6	1.0 *M* NH_4 phosphate	r	r	p	r	90
12. 0.2 *M* Mg chloride	Na HEPES pH 7.5	30%(*v*/*v*) 2-propanol	r	r	c	r	80
13. 0.2 *M* Na citrate	Tris–HCl pH 8.5	30%(*v*/*v*) PEG 400	c	c	p	c	0
14. 0.2 *M* Ca chloride	Na HEPES pH 7.5	28%(*v*/*v*) PEG 400	r	c	c	p	35
15. 0.2 *M* NH_4 sulfate	Na cadodylate pH 6.5	30%(*w*/*v*) PEG 8000	r	c	p	c	15
16. None	Na HEPES pH 7.5	1.5 *M* Li sulfate	r	c	p	c	25
17. 0.2 *M* Li sulfate	Tris–HCl pH 8.5	30%(*w*/*v*) PEG 4000	r	c	p	c	15
18. 0.2 *M* Mg acetate	Na cacodylate pH 6.5	20%(*w*/*v*) PEG 8000	r	d	c	r	75
19. 0.2 *M* NH_4 acetate	TrisHCl pH 8.5	30%(*v*/*v*) 2-propanol	r	d	c	d	70
20. 0.2 *M* NH_4 sulfate	Na acetate pH 4.6	25%(*w*/*v*) PEG 4000	r	d	c	p	70
21. 0.2 *M* Mg acetate	Na cacodylate pH 6.5	30%(*v*/*v*) MPD	c	c	c	p	0
22. 0.2 *M* Na acetate	Tris–HCl pH 8.5	30%(*w*/*v*) PEG 4000	r	c	c	p	35
23. 0.2 *M* Mg chloride	Na HEPES pH 7.5	30%(*v*/*v*) PEG 400	c	c	c	c	0
24. 0.2 *M* Ca chloride	Na acetate pH 4.6	20%(*v*/*v*) 2-propanol	r	d	c	p	70
25. None	Imidazole pH 6.5	1.0 *M* Na acetate	r	d	c	r	75
26. 0.2 *M* NH_4 acetate	Na citrate pH 5.6	30%(*v*/*v*) MPD	c	c	c	p	0
27. 0.2 *M* Na citrate	Na HEPES pH 7.5	20%(*v*/*v*) 2-propanol	r		c	c	70
28. 0.2 *M* Na acetate	Na cacodylate pH 6.5	30%(*w*/*v*) PEG 8000	r	c	c	c	50
29. None	Na HEPES pH 7.5	0.8 *M* K,Na tartrate	r	d	c	r	75
30. 0.2 *M* NH_4 sulfate	None	30%(*w*/*v*) PEG 8000	r	d	p	p	70
31. 0.2 *M* NH_4 sulfate	None	30%(*w*/*v*) PEG 4000	r	c	p	p	50
32. None	None	2.0 *M* NH_4 sulfate	r	d	p	c	85
33. None	None	4.0 *M* Na formate	c	c	p	c	0
34. None	Na acetate pH 4.6	2.0 *M* Na formate	r	c	p	r	50
35. None	Na HEPES pH 7.5	1.6 *M* Na, K phosphate	r	r	p	r	90
36. None	Tris–HCl pH 8.5	8% (*w*/*v*) PEG 8000	r	r	c	p	80
37. None	Na acetate pH 4.6	8% (*w*/*v*) PEG 4000	r	r	c	p	85
38. None	Na HEPES pH 7.5	1.4 *M* Na citrate	r	c	p	c	5
39. None	Na HEPES pH 7.5	2%(*v*/*v*) PEG 400 2.0 *M* NH_4 sulfate	r	d	p	c	50
40. None	Na citrate pH 5.6	20%(*v*/*v*) 2-propanol 20%(*w*/*v*) PEG 4000	r	c	p	p	5
41. None	Na HEPES pH 7.5	10%(*v*/*v*) 2-propanol 20%(*w*/*v*) PEG 4000	r	d	c	p	70
42. 0.05 *M* K phosphate	None	20%(*w*/*v*) PEG 8000	r	d	c	r	50
43. None	None	30%(*v*/*v*) PEG 1500	r	c	c	p	25
44. None	None	0.2 *M* Mg formate	r	r	c	r	90
45. 0.2*M* Zn acetate	Na acodylate pH 6.5	18% (*w*/*v*) PEG 8000	r	d	c	r	85
46. 0.2 *M* Ca acetate	Na cacodylate pH 6.5	18%(*w*/*v*) PEG 8000	r	r	c	p	80
47. None	Na acetate pH 4.6	2.0 *M* NH_4 sulfate	r	r	p	r	85
48. None	Tris–HCl pH 8.5	2.0 *M* NH_4 phosphate	r	r	p	r	90
49. 1.0 *M* Li sulfate	None	2%(*w*/*v*) PEG 8000	r	d	p	r	70
50. 0.5 *M* Li sulfate	None	15%(*w*/*v*) PEG 8000	r	d	p	r	70

[a] Percentages of saturated solutions at room temperature.
[b] Percentage of saturated solution necessary for glass formation.

Figure 1
Saturated lithium salt additions needed to suppress ice-ring formation in Hampton Screen Kit I solutions. (Abbreviations: c, clear; r, rings; d, diffuse; p, precipitate.)

3.2. Glass formation of crystallization solutions

The glass-forming properties of cryosalts were tested against the 50 mixtures that comprise the Hampton Screen Kit I (Hampton Research, Laguna Beach, CA, USA). The results of two different sets of experiments are shown in Fig. 1. In the first set, the cryoprotective behavior of the three salts lithium formate, lithium chloride and lithium sulfate were examined. Saturated solutions of each of the three lithium salts were mixed with equal volumes of the Hampton reagents and then flash-frozen. For mixtures that formed clear glasses, X-ray diffraction images were used to assess the presence of ice or salt-crystal formation. Many of the images were clear (c), but in some cases rings (r) were present or the image had a broad diffuse band (d) in the region where sharp rings appear. In many cases the mixture precipitated (p); for these, no diffraction experiments were performed.

Each of the three salts in these experiments were capable of forming glasses that are free of ice and salt microcrystals for many of the reagent mixtures. The results (Fig. 1) clearly illustrate the variability in the cryosalt behavior that should be expected because of the differences in the anions and the solubility properties of the salts (Table 1). Interestingly, none of the solutions with added lithium formate precipitated.

Concentrations of lithium formate necessary to inhibit rings were determined in a second set of experiments. With the exception of solutions 1, 5, 13, 21, 23, 26 and 33, which do not need cryoprotectants to form ring-free glasses upon freezing (Garman & Mitchell,

1996), for each solution a salt concentration was determined (rightmost column in Fig. 1) that would inhibit ring formation. At phosphate concentrations >0.4 *M*, an extremely high addition of lithium formate, (80–90% saturated lithium formate diluting the phosphate equivalently) was required for ring suppression.

3.3. Cryosalt use with protein crystals

The salts of Table 1 have proven to be effective cryoprotectants for X-ray data collection from crystals of biological macromolecules at cryogenic temperatures. For example, trigonal RNase S crystals grown from high salt (60% saturated ammonium sulfate and 25% saturated sodium chloride) are used to illustrate this point. Typically, data collection with frozen crystals has required treatment with a stabilizing solution that contains 30% glycerol as a cryoprotectant (Gilliland, Chu, Tordova & Ladner, unpublished data). In the absence of the cryoprotectant, the flash-frozen crystallization solution produces diffraction images containing rings, as shown in Fig. 2(*c*). These unwanted diffraction pattern features result from the presence of ice and ammonium sulfate microcrystals (compare Figs. 2*a*, 2*b* and 2*c*). Mixing the solution with two parts saturated lithium formate eliminates the presence of the rings, as is shown in Fig. 2(*d*). An RNase S crystal mounted in the solution containing the cryosalt produces the ring-free diffraction images illustrated in Fig. 3(*b*). A summary of recent data collection on a variety of proteins crystallized under different conditions is provided in Table 2. The resolution and data-analysis statistics are comparable with data sets collected from crystals using other cryoprotectants.

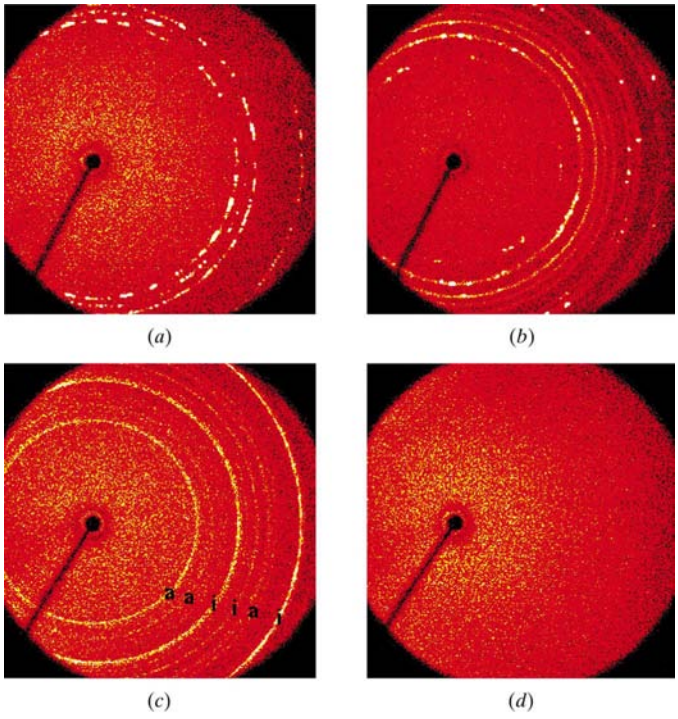
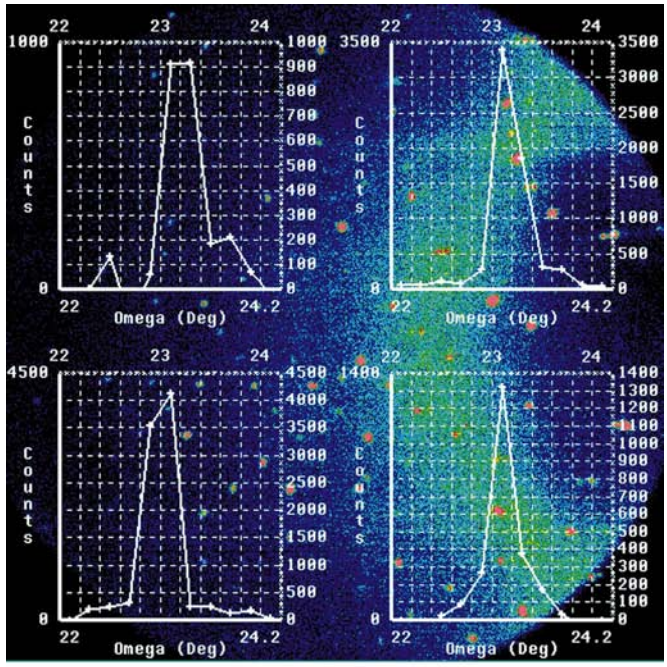


Figure 2
Area-detector diffraction images of frozen solutions maintained at 100 K: (*a*) for water, (*b*) supersaturated ammonium sulfate, (*c*) RNase S crystallization solution. The rings formed by the presence of ice and ammonium sulfate microcrystals are indicated with **i** and **a**, respectively. (*d*) RNase S solution:saturated lithium formate, 1:2.



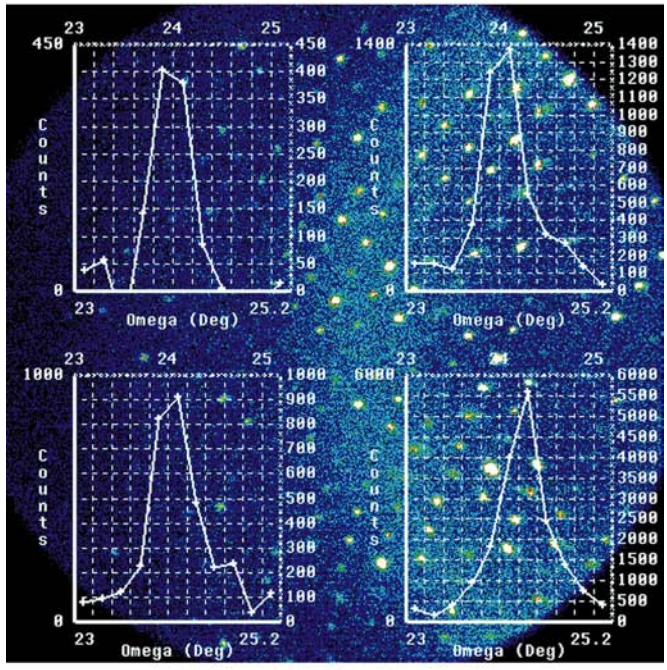


Figure 3
Area-detector diffraction images with overlays of randomly selected rocking curves are shown (*a*) for an RNase S crystal mounted in a capillary with the temperature controlled at 293 K and (*b*) for an RNase S crystal maintained at 100 K in a cryoprotectant solution of 2:1 saturated Li formate:crystallization solution. For both sets of data, the detector was positioned with 2θ at 30°, the ω-scan width for each image was 0.2°, the exposure time was 90 s and the crystal-to-detector distance was 10 cm.

Table 2
Protein crystal data collection using cryosalts at 100 K to suppress ice-ring formation.

Macromolecule	Well solution	Resolution† (Å)	Cryosalt
Lysozyme, hen egg-white	6.5% saturated NaCl, 100 m*M* NaOAc pH 4.8, 0.02% NaN_3	1.7	50% 2 *M* Li_2SO_4
Ribonuclease S	25% saturated $(NH_4)_2SO_4$, 60% saturated NaCl, 100 m*M* NaOAc pH 5.0	1.4	50% 2 *M* Li_2SO_4
Ribonuclease S	25% saturated $(NH_4)_2SO_4$, 60% saturated NaCl, 100 m*M* NaOAc pH 5.0	1.7	67% saturated $LiCHO_2$
HI0065, *Haemophilus influenzae*	1.6 *M* $(NH_4)_2SO_4$, 0.1 *M* Tris pH 7.2, 2.0% PEG 400	**2.3**	50% 2 *M* Li_2SO_4
HI1288, *Haemophilus influenzae*‡	0.6 *M* Na,K tartrate, 100 m*M* HEPES pH 7.1	**2.6**	75% saturated $LiCHO_2$
HI1715, *Haemophilus influenzae*‡	2.3 *M* $(NH_4)_2SO_4$, 100 m*M* HEPES pH 7.1	**2.1**	88% saturated Li_2SO_4
β lactamase, *Staphylococcus aureus*§	84% saturated $(NH_4)_2SO_4$, 100 m*M* $NaHCO_3$ pH 8.0, 0.5% PEG 2000	1.9	90% saturated Li_2SO_4, 100 m*M* $NaHCO_3$ pH 8.0

† Resolution limits shown in bold type are for data collected at the Argonne National Laboratory APS. ‡ N. Bonander & G. L. Gilliland, unpublished results. § C. C. H. Chen & O. Herzberg, unpublished results.

3.4. Cryosalt addition

Salts added to the crystallization medium act synergistically with uncharged organics, such as PEGs, mono and polyalcohols, as well as the ionic species already present. Because of these factors and the dependence of glass formation on the freezing rate and on the stability of the ice crystal, rigid numerical guidelines which dictate which salt should be added and at what concentration are impractical. This complexity is indicated in Fig. 1, which illustrates how the effectiveness of cryosalt ice suppression depends on all the components of the crystallization solution.

The following rules are suggested for selecting which salt to use to suppress microcrystalline ice and salt-crystal precipitation.

(i) To lower stresses on the crystal, use a salt solution that has an anion or cation in common with the mother liquor/precipitating agent if possible.

(ii) Check for precipitation in the solution at ambient temperature. It is not uncommon for highly concentrated salt solutions of mixed species – such as those commonly used as crystallizing agents – to form precipitates or phase separations.

(iii) Leave the mixed solution in contact with the crystal for about 10 s to allow equilibration through the unstirred layer to the crystal surface before freezing.

(iv) Lithium solutions can be incompatible with phosphates since together they tend to form relatively insoluble precipitates.

The ratio of saturated cryosalt to the crystal-stabilization solution necessary to obtain a glass is easiest to determine separately prior to mounting the crystal. It is best to start with a 1:1 mixture of cryosalt to stabilization solution.

3.5. Cryosalt impact on mosaicity and diffraction resolution

Flash-freezing, physical manipulation and chemical treatment can all contribute to changes in mosaicity. As pointed out by Garman (1999), with traditional cryoprotectants the mosaic spread of rapidly frozen crystals often becomes larger than that observed for comparable room-temperature crystals. Better behavior appears to hold true for crystals treated with cryosalts. In Fig. 3, the reflection's rocking-curve profiles (which depend on the mosaicity) from an untreated RNase S crystal at room temperature and from a flash-frozen RNase S crystal soaked in mother liquor containing lithium formate are compared. The profiles in both cases have a full-width at half-height of 0.5–0.6°. Thus, in this case and others tested so far mosaicity changes upon freezing with cryosalts are minimal, allowing X-ray data collection and data processing to proceed normally.

Cryosalts, like traditional cryoprotectants, increase the diffraction resolution above that observed for wet mounted crystals, where the X-ray intensities are attenuated by capillaries. As yet, no decrease in diffraction resolution has been observed for any of the macromolecular crystals from which data have been collected. Although not yet systematically studied, it is expected that cryosalts will prove as effective at crystal annealing (Harp *et al.*, 1998) or flash-annealing (Yeh & Hol, 1998) as the organic cryoprotectants reported earlier.

3.6. Cryosalts: general considerations

A number of transient changes occur across the crystal–solution interface when the crystal is exposed to the cryoprotectant solution. The transients in osmotic pressure and interface electrochemical potential arise from the differences in transport rates, primarily diffusion, between the various species on opposite sides of the interface. It is impossible to match two salt solutions so that they have the same effective ionic properties and osmotic properties at the same time. To a first approximation, colligative properties depend on the number of ionic and molecular particles in the water. Unless one wants to change the water content of the crystal, it is worthwhile to balance the osmotic pressures. However, it is better to be too concentrated than too dilute in the added salt. Dilute solutions tend to increase the hydraulic pressure inside the crystal, which may lead to fractures and the ultimate destruction of the crystal. Of course, a number of steps having smaller changes that gradually increases the cryosalt concentration may be more effective at reducing the stresses. It should be noted that the transient interface electrochemical potential that arises has its effects at long distance quenched

by the high levels of salt. However, its local effects on crystal stresses are unknown.

4. Conclusions

The aqueous glass-forming properties of highly soluble salts have been known for many years. Nevertheless, these compounds have not been reported as cryoprotectants for macromolecular crystallography. Cryosalt addition to commonly used crystallization solutions and protein crystals indicate their glass-forming ability under conditions of utility for cryocrystallography, with attributes comparable to the traditional organic cryoprotectants. In addition, the absence of deleterious effects on mosaicity and diffraction resolution of cryosalt-treated crystals make them potentially as useful as the more traditional cryoprotectants. Cryosalts, by themselves or in combination with traditional cryoprotectants, may offer an advantage for macromolecular crystals grown in the presence of high salts. Because of the variety of salts that appear to be useful (*e.g.* Table 1 and others to be reported), finding a close match of the appropriate cryoprotectant with the crystallization salt properties (electrostatic and colligative) may prove to be advantageous for systems that prove difficult to freeze with traditional cryoprotectants. Thus, cryosalts expand the options for producing crystal samples suitable for cryocrystallographic data.

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