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Cryoprotection properties of salts of organic acids: a case study for a tetragonal crystal of HEW lysozyme

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Currently, the great majority of the data that are used for solving macromolecular structures by X-ray crystallography are collected at cryogenic temperatures. Selection of a suitable cryoprotectant, which ensures crystal stability at low temperatures, is critical for the success of a particular diffraction experiment. The effectiveness of salts of organic acids as potential cryoprotective agents is presented in the following work. Sodium formate, acetate, malonate and citrate were tested, as were sodium potassium tartrate and acetate in the form of potassium and ammonium salts. For each salt investigated, the minimal concentration that was required for successful cryoprotection was determined over the pH range 4.5–9.5. The cryoprotective ability of these organic salts depends upon the number of carboxylic groups; the lowest concentration required for cryoprotection was observed at neutral pH. Case-study experiments conducted using the tetragonal form of hen egg-white lysozyme (HEWL) confirmed that salts of organic acids can successfully act as cryoprotective agents of protein crystals grown from high concentrations of inorganic salts. When crystals are grown from solutions containing a sufficient concentration of organic acid salts no additional cryoprotection is needed as the crystals can safely be frozen directly from the crystallizing buffers.

1. Introduction

In the vast majority of cases, X-ray diffraction data from crystals of biologically important macromolecules are collected at cryogenic temperatures. Methods for collecting such data using cryocrystallography were first introduced in the 1980s (Hope, 1988). The percentage of X-ray data collected at low temperatures has dramatically increased in the past 15 years. In 1995, only 25% of all solved structures were determined at cryotemperatures. In the next five years this fraction more than tripled, increasing to 77% (Chruszcz *et al.*, 2008). By 2009, 97% of all experiments reported by the PDB were conducted at cryogenic temperatures. Nowadays, the development of new cryo-techniques and cryo-protocols is in great demand and thus takes place at a very rapid pace. Cryocrystallography offers a great variety of advantages, namely reduction of X-ray-induced radiation damage, methodological improvements and greatly facilitated structure solution (Garman & Schneider, 1997). Application of low-temperature procedures allows a significant reduction of the destructive effects induced by X-ray radiation and at the same time enhances the stability of crystals of biomacromolecules (Burmeister, 2000). This feature permits the use of very high-

flux radiation sources such as synchrotrons, as well as the opportunity to collect multiple complete data sets from one crystal in MAD experiments (González, 2003). Moreover, the results obtained during cryogenic X-ray diffraction experiments are of better quality compared with room-temperature data, because cryocooling significantly decreases the rate of crystal degradation arising from radiation damage. Additionally, data sets that display uniform quality throughout the whole period of data collection are easier to process and give better statistics.

Collecting diffraction data at temperatures below 150 K allows significant methodological improvements (Garman & Owen, 2007). Among these, facilitated halide derivatization (Dauter *et al.*, 2000, 2001) and the possibility of harvesting crystals while they are under optimal conditions (especially for crystals that deteriorate soon after growth) are of primary importance. Cryo-techniques allow the long-term storage of samples, which is of enormous benefit to home laboratories and is advantageous both practically and economically. Additionally, the initial diffraction quality of crystals can be checked using laboratory X-ray sources and the best crystals can subsequently be retrieved for complete measurement on synchrotron beamlines.

Collecting diffraction data at low temperatures is also advantageous for structure solution. Owing to the diminished intermolecular motion and decreased thermal vibration at temperatures below 150 K, the measured diffraction images are frequently of better quality compared with those collected at room temperature, which facilitates model building and refinement (Halle, 2004). Furthermore, cryocrystallography makes it possible to trap the intermediates of enzymatic reactions in some cases (Westenhoff *et al.*, 2010), *e.g.* in dethiobiotin synthesis (Käck *et al.*, 1998) and peptide-bond hydrolysis (Wilmouth *et al.*, 2001).

During experimental procedures at low temperatures, the formation of microcrystalline ice within biological specimens is the most undesirable obstacle. The first problem connected with ice formation is the considerable damage that is created owing to the mechanical stress that accompanies the phase transition of water from liquid to hexagonal ice. This phenomenon can damage the integrity of the diffraction pattern, causing smearing and splitting of the observed reflections. Secondly, the presence of 'ice rings', diffraction rings arising from the powder diffraction of the microcrystalline ice, influences data processing. Since the ice rings occlude some reflections from the crystal, the resulting data sets that are collected are not complete. The region covered by the ice rings roughly corresponds to the reflections of a resolution near the length of hydrogen bonds. The data with a resolution near the typical hydrogen-bond length are crucial for proper structure solution and refinement. The presence of ice rings is a major cause of error in refined models.

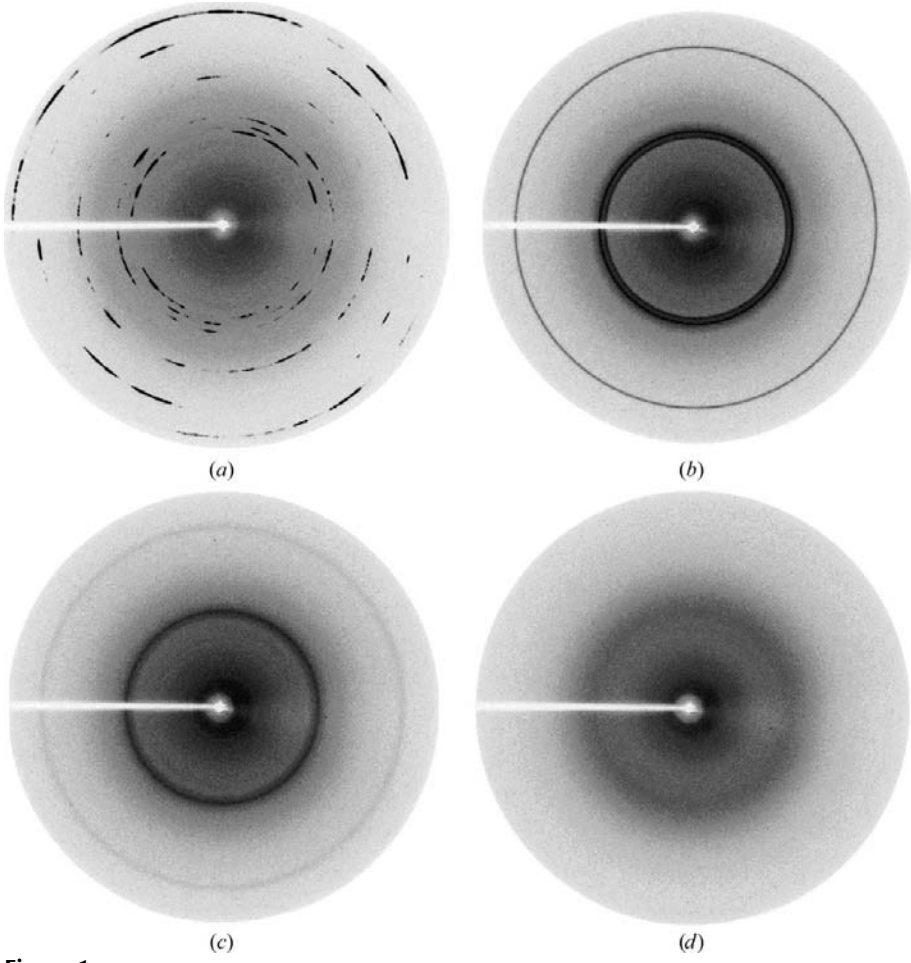

Figure 1
The effects of cryoprotection. (*a*) The presence of 'ice rings' indicates a lack of cryoprotection. (*b*) The background scattering is very high (the ice rings are of intensity $>10\sigma$), indicating that the cryo-salt concentration is too low. (*c*) Poor cryoprotection is represented by dark ice rings. This indicates a need for an increase in the cryoprotectant concentration. (*d*) The intensity of the ice-ring pattern is less than two times the background, indicating successful cryoprotection.

Difficulties with ice formation within a protein crystal can be overcome by the use of a suitable cryoprotectant. A versatile antifreeze agent, which can be used for most macromolecular crystals, would be the best solution and would significantly facilitate experiments (Young *et al.*, 1994; Kurinov & Harrison, 1995). The selection of a proper cryoprotectant is undoubtedly required for success of the whole experiment. However, in practice finding a suitable antifreeze agent can be troublesome and extremely time-consuming, delaying data collection. A mismatched cryoprotectant can cause distortion, dissolution or breakage of the crystal. The effects of poor cryoprotection *versus* good cryoprotection are clearly depicted in Fig. 1.

Table 1
Salts of organic acids investigated in this work.

Salt	Formula	Molecular weight
Sodium formate	HCOONa	68.01
Ammonium acetate	CH_3COONH_4	77.08
Sodium acetate	CH_3COONa	82.03
Potassium acetate	CH_3COOK	98.15
Sodium malonate	$CH_2(COONa)_2$	148.03
Sodium citrate	$C_6H_5O_7\ Na_3$	258.07
Sodium potassium tartrate	$C_4H_4O_6KNa$	282.1

Five main groups of compounds are used as cryoprotectants: alcohols [*e.g.* methanol, ethanol, 2-propanol, methylpentanediol (MPD), ethylene glycol, glycerol], monosaccharides and disaccharides (*e.g.* glucose, fructose and galactose), low-molecular-weight polymers such as polyethylene glycol (PEG), mineral oils (which in fact are not true cryoprotectants, although they do enable sample vitrification without the formation of ice rings) and salts, both organic acid salts (Holyoak *et al.*, 2003) and, in rare cases, inorganic salts (Rubinson *et al.*, 2000).

Interest in carboxylic acid salts as possible cryoprotectants has increased significantly over the past few years. The first report discussing cryoprotection using a carboxylic acid salt was published only quite recently (Holyoak *et al.*, 2003). The cryoprotective effectiveness of malonate was verified by cryocooling five enzyme samples in its solutions. However, this publication does not suggest the concentrations or pH levels that should be applied in order to cryoprotect new macromolecular crystals in sodium malonate solutions. In parallel, the practical use of sodium malonate as both a crystallization agent and a cryoprotectant for the PX domain of CISK has been reported (Xing & Xu, 2003). The use of sodium citrate in the crystallization and cryoprotection of plant pathogenesis-related protein PR-10 and cytokinin-specific binding proteins has been reported (Bujacz *et al.*, 2003; Pasternak *et al.*, 2005, 2006; Fernandes *et al.*, 2008, 2009).

In the current work, we found that a number of organic carboxylic acid salts (Table 1), among them sodium malonate, are effective cryoprotectants. The tetragonal crystal form of HEW lysozyme was used as a case study to check their cryoprotective properties. Additionally, the ability to form a vitrified drop was proven by X-ray diffraction procedures conducted at low temperatures over a wide pH range. Crystals grown using high concentrations of mineral salts as precipitants often present a challenge in that they are difficult to cryoprotect. Crystals precipitated from chlorides, sulfates or fluorides often crack or dissolve upon transfer from mother liquor into glycerol-containing buffers (Harp *et al.*, 1998; Garman & Owen, 2007). In such cases, the method of choice would be transfer of the crystals to organic acid salt solutions such as those investigated in this work.

The main aim of the experimental procedure was to determine the minimal concentration of each salt that provided satisfactory cryoprotection. The pH level was investigated as a secondary parameter of the minimum concentration, as the pH of the cryoprotectant should be similar to the pH of the crystallization buffer in order to avoid changes in the surface properties of the protein. Such pH alterations can cause crystal damage and cracking. A further goal was to prove that solutions of carboxylic acids could provide excellent cryoprotection for salt-grown macromolecular crystals without the addition of glycerol, which is the cryoprotectant used in most commercial screens, such as Hampton Research Crystal Screen Cryo (Garman & Mitchell, 1996).

2. Materials and methods

2.1. Cryoprotectants

The salts of carboxylic acids (Table 1) were selected on the basis of the experimental experience of the authors. Since the popularity of the use of organic acid salts in protein crystallization has recently increased (McPherson, 2001), we considered it worthwhile to prove that such crystallization buffers could also be good cryoprotectants by themselves. Cryoprotectant buffers for data collection at different pH levels were prepared by mixing stock solutions of salts of organic acids with different buffers within the pH range 4.5–9.5, adding the necessary amounts of water. In the experiments described here, the following stock buffers of concentration 1 *M* were used: sodium acetate pH 4.5, sodium citrate pH 5.5, MES pH 6.5, HEPES pH 7.5, Tris pH 8.5 and Bicine pH 9.5. The concentration of the above buffers in the obtained cryosolutions used during diffraction experiments was 0.2 *M* and the final pH values are reported in Table 2. The additional amounts of acetate and citrate anions have to be considered during practical application of this method when other buffers are used at acidic pH.

2.2. Scope and realisation of the experiment

The cryoprotective properties of each carboxylic acid salt were examined in two independent experiments under the same conditions, according to similar procedures. As stated earlier, one experimental aim was to obtain the minimal salt concentration needed for successful cryoprotection as a function of pH. Salt solutions of varying concentrations were picked up using a nylon loop with a diameter of 0.25 mm and then flash-cooled directly in a nitrogen-gas stream from a low-temperature unit set at 100 K. The frozen droplet of cryosolution was exposed to X-ray radiation for 2 min to collect a diffraction image. The procedure was repeated over the entire pH range (4.5–9.5) with unit pH intervals. The concentration of salt for which the intensity of the ice ring was less than twice that of the background scattering was assumed to be the minimal salt concentration needed for successful cryoprotection (Fig. 2). In order to make the procedure easier, this relationship was expressed in terms of σ, the intensity of the background scattering. Using the option to display an intensity profile in the *XDISP* program (Otwinowski & Minor, 1997), it was possible to calculate the ratio $I_{\rm ring}$ of the strength of the ice diffraction ring to the background intensity. In other words, when $I_{\rm ring}$ was greater than 2 the particular concentration of

Table 2
The minimum salt concentration required for successful cryoprotection by organic acid salts.

The buffer stock solutions have 1 *M* concentration and the final concentration of the buffer in the cryoprotective solutions was 0.2 *M* (the actual pH value pH is given in parentheses).

	Buffer					
Salt	Acetate pH 4.5	Citrate pH 5.5	MES pH 6.5	HEPES pH 7.5	Tris pH 8.5	Bicine pH 9.5
Sodium formate	4.00 (5.31)	3.95 (5.98)	3.60 (6.91)	3.00 (7.61)	3.75 (8.80)	3.60 (9.36)
Ammonium acetate	3.80 (5.70)	3.40 (6.42)	3.40 (7.09)	3.40 (7.65)	3.80 (8.36)	3.80 (8.87)
Sodium acetate	3.95 (5.65)	2.70 (6.38)	2.55 (6.85)	2.40 (7.59)	2.65 (8.76)	2.70 (9.41)
Potassium acetate	2.90 (5.61)	2.80 (6.34)	2.80 (7.82)	2.60 (7.56)	2.90 (8.75)	2.80 (9.48)
Sodium malonate	2.20 (5.59)	2.10 (6.32)	1.95 (7.05)	1.80 (7.69)	2.35 (8.91)	2.60 (9.46)
Sodium citrate	1.50 (5.61)	1.20 (6.27)	1.15 (7.01)	1.15 (7.65)	1.30 (8.97)	1.30 (9.49)
Sodium potassium tartrate	2.20 (5.26)	2.00 (6.02)	2.00 (6.98)	1.90 (7.72)	1.90 (9.02)	1.90 (9.61)

antifreeze agent was considered to be insufficient for cryoprotection of a macromolecular crystal at a particular pH. In most instances, we tried to find the lowest value of the cryoprotectant concentration resulting in the best cryoprotective effect. For one salt, sodium acetate, we also checked the influence of loop size. For small loops with diameters of 0.05–0.25 mm we did not observe any correlation between loop size and the concentration of salt required for successful cryoprotection. Larger loops with diameters of 0.4–0.6 mm required an increase of the salt concentration by 0.05 *M* at pH 6.5 and 7.5 and around 0.1 *M* for basic and acidic pH, which confirms that the speed of cooling is one of the important factors in successful cryoprotection (Angell & Choi, 1986). The influence of sample preparation was also investigated by dipping the mounting loop in liquid nitrogen for the case of sodium acetate and the same concentration was obtained as when stream cooling was applied. This method was more efficient for larger loop sizes and did not require an increase in salt concentration when increasing the loop size.

The second experiment involved a series of X-ray diffraction measurements of HEW lysozyme crystals cryoprotected using the investigated salts at the crystallization pH. The HEW lysozyme crystals were grown from 1.4 *M* NaCl in sodium acetate buffer pH 4.5. We used similarly sized crystals of 0.15–0.2 mm mounted in the same-sized loop with a diameter of 0.25 mm. The crystals were subjected to X-ray radiation after being cooled in particular cryoprotectants at concentrations corresponding to the minimal salt concentrations determined in the first experiment for acetate buffer at pH 4.5. It is worth noting that the investigated salts are less effective cryoprotectants at acidic pH than at neutral pH.

The goal of the research was to investigate a new cryoprotocol based on salts of organic acids for low-temperature diffraction experiments using HEW lysozyme crystals. This approach can be used for crystals grown in organic salt buffers and for crystals obtained from inorganic salts when cryoprotection by polyalcohols was unsuccessful or insufficient. In order to compare the new approach with traditional cryoprotectants, a control experiment using 20% glycerol as an

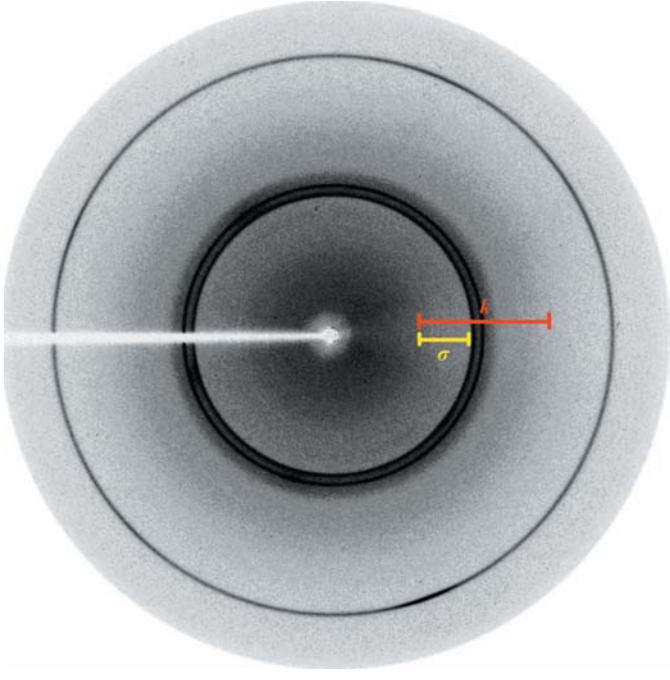

Figure 2
Graphical representation of the method used for calculation of the relation between ice-ring intensity (k) and background scatter (σ). $I_{ring} = k/\sigma$.

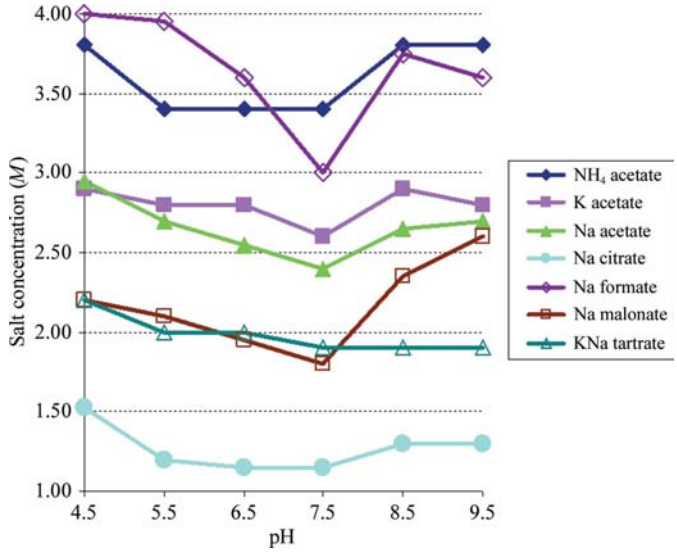

Figure 3
Comparison of the minimal concentration profiles obtained for seven carboxylic acid salts in the pH range 4.5–9.5.

antifreeze agent added to the mother liquor (1.4 *M* NaCl and 0.1 *M* sodium acetate pH 4.5) was performed.

2.3. X-ray diffraction data collection

The measurements were carried out on a MAR 300 detector using graphite-monochromated Cu *Kα* radiation. The stream of cold nitrogen (100 K) was covered with a plastic shield just before placing the loop on the goniometer head in order to avoid variations in the rate of cryocooling of the samples. Between 14 and 40 images were collected on average for each cryosalt, with an exposure time of 600 s for a single frame of 1° oscillation; the detector distance of 150 mm resulted in a data resolution of 2.05 Å. Investigation of the images allowed the calculation of the mosaic spread, which served as an indicator of cryoprotection quality. Additionally, differences in unit-cell parameters were analyzed. The collected diffraction images were indexed and integrated with *DENZO* and the intensity data were scaled with *SCALEPACK* in the current version of the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

The minimal antifreeze agent concentration that resulted in successful cryoprotection was determined for a variety of carboxylic acid salts. A diagram showing the minimal salt concentration required for successful cryoprotection at various pH values is shown in Fig. 3; numerical data and actual pH values are given in Table 2. Analysis of the gathered data definitively proved the effectiveness of all the investigated salts as cryoprotectants over the entire pH range (pH 4.5–9.5).

3.1. The relationship between the degree of cryoprotection and the number of carboxylic groups

Our main conclusion concerns the relationship between the number of carboxylic groups that are present in the organic acid and the minimum concentration that is required for successful cryoprotection by each compound. The correlation is inverse and is clearly noticeable in Fig. 3. In other words, the minimal cryoprotectant concentration decreases with an increase in the number of carboxylic groups in the organic acid. Sodium citrate, which is the salt of a tricarboxylic acid, provides cryoprotection at the lowest concentrations. Sodium malonate and sodium potassium tartrate, both of which possess two carboxyl groups, display cryoprotective ability at similar concentrations. For the two salts with a single carboxylic acid group, acetate exhibits better cryoprotective properties than formate. Sodium formate required the highest concentration of the sodium salts to effectively prevent ice formation. The specific choice of cryoprotective salt in an experiment should take into account not only the concentration required, but also other factors such as the chelation of metal cations, as is known for citrate.

3.2. The influence of pH on the minimal cryoprotectant concentration required for the proper cryoprotection of protein crystals

For all the cryo-salts tested, pH values close to neutral required the lowest concentrations for successful cryoprotection (Fig. 4). Any change in pH from 7.5 to either higher or lower pH increased the minimum salt concentration required. This dependence is especially visible for potassium acetate and for sodium malonate, acetate and formate. In contrast, the pH dependencies for sodium citrate and sodium potassium tartrate are rather flat, suggesting that the cryoprotective effects of both compounds are maximally effective over a large pH range. The lowest minimal concentrations required for tartrate are found at pH 7.5–9.5. For some of the cryoprotectants tested, namely sodium citrate and ammonium acetate, concentration minima are observed for a wide range of pH values below the neutral point.

The minimal concentrations required for cryoprotection by each salt varied considerably. In general, sodium citrate displayed the lowest minimal concentrations at all pH values, even at pH 7.5 (Fig. 3). This indicates the very effective cryoprotective properties of this salt. In contrast, ammonium

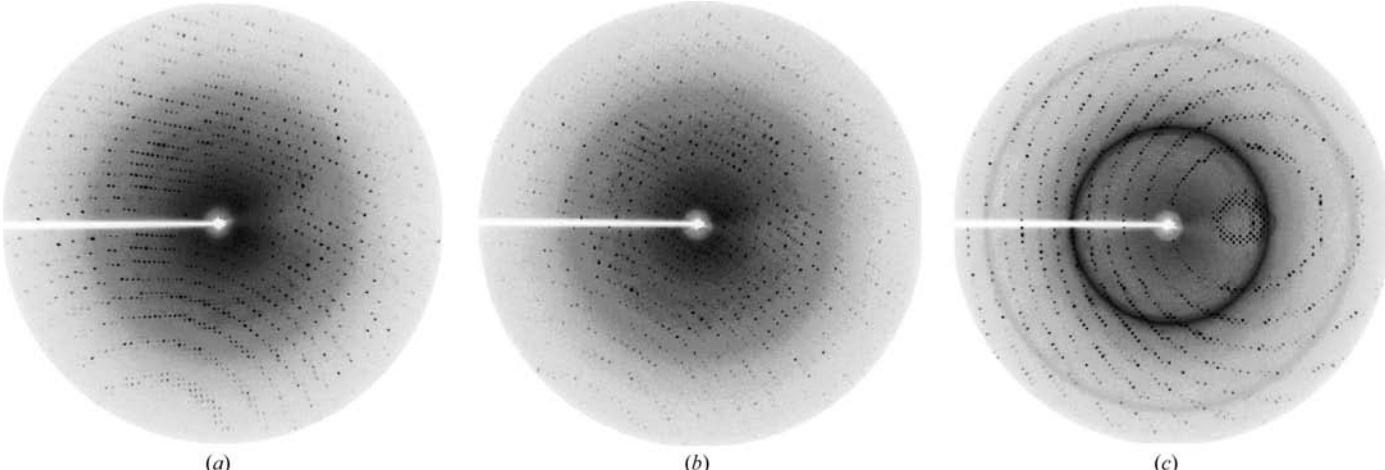

Figure 4
(*a*) Diffraction image of a lysozyme crystal cryoprotected using 4.0 *M* sodium formate solution at pH 4.5. (*b*) Diffraction image of a lysozyme crystal cryoprotected using 1.5 *M* sodium citrate solution at pH 4.5. (*c*) Diffraction image of a lysozyme crystal cryoprotected using 20% glycerol solution.

acetate required the highest minimal concentrations, especially at neutral and basic pH values. Even at pH 7.5, at which the minimal concentration for ammonium acetate reached its minimum, 3.4 *M* of the compound was required for cryoprotection. However, the data indicate that sodium acetate, formate, malonate, citrate and sodium potassium tartrate, as well as ammonium acetate and potassium acetate, can successfully act as cryoprotective agents at pH values in the range 4.5–9.5.

3.3. The influence of the cation on acetate salts

Different cations display different hydrolytic properties (Chipman & Chen, 2006). Since the cation influences the cryoprotective properties of a given salt of a particular organic acid, three cations were tested in this experiment. The results revealed an interesting correlation between the minimal cryo-salt concentration for cryoprotection and the cation used. Sodium and potassium cations are regarded as highly hydrophilic, meaning that they readily attract water molecules and thus usually interact only weakly with proteins (Chipman & Chen, 2006; Uejio *et al.*, 2008). For this reason, these ions are known as 'hard' cations. In contrast, ammonium is 'soft', meaning that it is more able to interact with negatively charged protein-surface patches and has a lesser tendency to be coated with water molecules (Annunziata *et al.*, 2006). It is evident that replacing a 'hard' cation by a 'soft' cation in a cryo-salt increases the concentration of the salt that is required for successful cryoprotection. Specifically, when sodium is exchanged for ammonium as the cation for an acetate salt, the minimal salt concentration increases by 1 *M* (Fig. 3). However, exchanging 'hard' cations does not significantly influence the cryoprotective ability of organic salts. The sodium and potassium acetate salts display similar cryoprotective properties; the difference in minimal salt concentration between the two is only 0.2 *M*.

3.4. The influence of additional hydroxyl groups on the cryoprotection level

Comparing the data obtained for all the investigated salts, the profiles of sodium citrate and sodium tartrate are flat at basic pH values. Owing to this behaviour, these two salts are the most effective cryoprotectants at pH values of 8.5 and 9.5 (Fig. 3). Both compounds are polycarboxylic acid salts and both contain hydroxyl groups. The presence of hydroxyl groups in sodium citrate and sodium tartrate may be responsible for the flattening of the minimal concentration profile at basic pH values.

3.5. The number of water molecules interacting with a single cryoprotectant molecule

At the molecular level, the active groups of the cryoprotectant create diverse interactions with water molecules. The diversity of the contacts arises from the variety in the chemical nature of the interactions and the different orientations of the interacting molecules. The groups of the cryoprotectant can act as proton donors, proton acceptors and polar contacts with water dipoles. Owing to disturbance of the water structure, the cryoprotectant molecule prevents ice-crystal formation during cryocooling. It is interesting to calculate the number of water molecules that interact with a single cryoprotectant molecule in solutions which can vitrify without ice formation. In a very popular cryoprotectant solution, 20%(*w*/*w*) glycerol in water, a single glycerol molecule is on average associated with 16.2 water molecules. Polyalcohols with multiple hydroxyl groups are the most commonly used cryoprotectants; additionally, they are conformationally labile, which increases the diversity of their interactions. The carboxylic acid salts interact with water molecules. Carboxyl groups in the ionized form are acceptors of protons and in the protonated form can interact as both donors and acceptors. The partner cations are hydrolyzed and because of this salts of carboxylic acids in a suitable concentration range disturb regular ice-crystal formation and act as cryoprotectants. We calculated the number of water molecules that interact with a single cryoprotectant molecule for all the investigated salts at pH 7.5. For the salts of organic acids with a single carboxyl group, one salt molecule is sufficient to prevent ice formation in a mixture with 16.6 water molecules for sodium formate and 20.2 water molecules for sodium acetate. Exchanging the sodium cation for potassium or ammonium decreases the cryoprotective ability and single molecules of the acetate salts associate with 17.9 and 12.7 water molecules, respectively. The salts of organic acids with two carboxyl groups are better cryoprotectants and prevent ice-crystal formation in a mixture with water in a molar ratio of 1:26.4 and 1:24.7 for sodium malonate and sodium potassium tartrate, respectively. The sodium salt of citric acid has the best cryoprotective properties and only 1 mol of this salt mixed with 39.5 mol water is still a good cryoprotectant. The organic acid salts with multiple carboxyl groups and additional hydroxyl groups show a conformational flexibility similar to that of polyalcohols which may increase their cryoprotective properties.

3.6. Diffraction data collected for lysozyme crystals

Experiments conducted on lysozyme crystals grown in 1.4 *M* NaCl confirmed the fact that sodium acetate, formate, malonate and citrate and sodium potassium tartrate, as well as ammonium acetate and potassium acetate, can successfully act as cryoprotective agents for protein crystals. All of the compounds diminish the possible crystal disruption arising from ice lattice formation.

The examined protein crystals behaved exactly as expected. None of the cryoprotectant solutions caused crystal cracking or dissolution on transfer from mother liquor to cryoprotectant buffer. In fact, the transfer of crystals from one salt to another was less harmful to the crystals than transfer to a glycerol-based cryoprotectant solution (Table 3). This observation strongly suggests that this cryoprotection method is not only suitable for crystals grown from carboxylic acid salts but may also be successfully applied to crystals grown from other mineral salts. In all cases, the diffraction patterns were of high quality with single sharp reflections, proving the effectiveness

Table 3
Summary of X-ray diffraction data collected from lysozyme crystals soaked in different cryoprotectants.

a, *b* and *c* are the unit-cell parameters; *V* is the unit-cell volume; $I/\sigma(I)$ is the mean intensity divided by the mean intensity error. Values in parentheses are for the highest resolution shell.

Cryoprotectant	$a = b$ (Å)	c (Å)	V (Å^3)	Mosaicity	$R_{\rm merge}$	$I/\sigma(I)$
Sodium formate	77.35	37.65	225300	0.53	0.042 (0.126)	21.3 (8.0)
Ammonium acetate	76.53	37.19	217800	0.65	0.034 (0.141)	24.5 (7.0)
Sodium acetate	78.08	37.03	225700	0.55	0.026 (0.078)	33.0 (11.7)
Potassium acetate	77.11	37.26	221600	0.51	0.047 (0.099)	15.0 (7.2)
Sodium malonate	76.63	37.29	219000	0.49	0.031 (0.082)	31.9 (11.6)
Sodium citrate	77.03	37.26	221100	0.56	0.048 (0.145)	17.8 (7.1)
Sodium potassium tartrate	77.24	37.41	223200	0.70	0.052 (0.271)	17.1 (3.5)
Glycerol	77.46	37.24	223500	0.83	0.043 (0.151)	33.5 (7.7)

of the studied salts in the cryoprotection of HEW lysozyme crystals grown from sodium chloride. None of the patterns displayed ice powder diffraction rings, which also supported the argument for the cryoprotective properties of the studied organic acid salts.

According to the data shown in Table 3, sodium malonate appears to exhibit very good cryoprotective properties as measured by the low mean mosaic spread. Conversely, the crystal for which glycerol was used as a cryoprotectant had the highest mean mosaicity. The second highest mean mosaicity (0.70°) was measured in the sodium tartrate buffer. This may be accounted for by the fact that 2 *M* tartrate is close to the salt's saturation point and rapid mounting was required in order to avoid its precipitation from the solution. The high concentration may have also caused other problems such as micro-cracking during cryocooling of the sample. A relatively high average mosaicity was also recorded for ammonium acetate. As described earlier, the presence of a 'soft' cation slightly lowers the cryoprotective abilities of the salt, which may explain this result. For the remaining salts, the mean mosaicity values attain good levels, indicating the high quality of the crystals under investigation. It is worth commenting that the beam divergence on a standard home generator with a graphite monochromator is higher than that on synchrotron beamlines and is included in the values of the mosaicity estimated by the data-processing software.

Several factors influence the changes in unit-cell volume observed for the different cryo-salts. Of these, the salt concentration and time of soaking seem to be of the greatest importance, although no general rule has been observed. In many instances, high salt concentrations cause water withdrawal from the crystal, leading to crystal dehydration, which could be the reason for crystal disruption in the extreme cases. On the other hand, when some crystals are kept in the salt solution for a longer period of time they shrink, attaining a more compact structure that results in higher quality X-ray diffraction data. To avoid the influence of soaking in high-salt solutions crystals were transferred through the cryoprotectants very quickly. For all the salts, $R_{\rm merge}$ is below or close to 5%, indicating that the collected data were of excellent quality.

The results presented here demonstrate the excellent cryoprotective abilities of carboxylic acid salts without additional glycerol (Figs. 4*a* and 4*b*). Some conditions from commercial crystallization screens designed to contain cryoprotectants, such as Hampton Research Crystal Screen Cryo, contain glycerol or other organic compounds even when they are not necessary. For example, the condition of Crystal Screen Cryo that contains 3.6 *M* sodium formate also contains 10%(*v*/*v*) glycerol. However, the data collected in this experiment prove that 3.0 *M* sodium formate is a sufficient cryoprotectant in HEPES buffer at pH 7.5 (or 4.0 *M* in acetate buffer at pH 4.5) with no need for the addition of glycerol. Similarly, the screen condition containing 1.26 *M* sodium citrate also contains 10%(*v*/*v*) glycerol, despite the fact that cryocooling protein crystals in a solution of 1.15 *M* sodium citrate alone assures effective cryoprotection in HEPES buffer pH 7.5 (or 1.5 *M* in acetate buffer pH 4.5; Pasternak *et al.*, 2005, 2006).

As a control, a lysozyme crystal grown from 1.4 *M* NaCl was vitrified in a solution containing 20%(*v*/*v*) glycerol. The diffraction images collected in this case showed the poorer cryoprotective properties of glycerol. The ice-ring intensity was much higher than the background intensity and the protein crystal diffraction pattern overlapped with the ice powder diffraction. Moreover, the presence of traces of 'ice rings' revealed the presence of microcrystalline ice and thus poor cryoprotection (Fig. 4*c*). This poor cryoprotection was further reflected by the high mean mosaicity measured for this crystal.

4. Conclusions

There are numerous advantages to using carboxylic acid salts as cryoprotectants for protein crystals. Firstly, as proved by the results shown here, several salts of carboxylic acids may serve as alternative cryoprotectants for salt-grown macromolecular crystals during X-ray diffraction data collection at low temperatures at a variety of pH values. Secondly, the investigated compounds can successfully be used as precipitating agents and such crystallization buffers can serve as cryoprotectants after harvesting crystals from crystallization drops (Bujacz *et al.*, 2003; Pasternak *et al.*, 2005, 2006; Fernandes *et al.*, 2008, 2009). When the concentration of a salt of an organic acid is not sufficient for cryoprotection, the safest solution to the problem and the lowest stress for the crystal is to increase the salt concentration of the reservoir (in a hanging-drop vapour-diffusion experiment) and wait a few days for the concentration of the salt in crystallization drop to slowly increase and allow the crystal to stabilize.

When sulfates or phosphates are used as precipitating agents, it is usually difficult to obtain heavy-metal derivatives in order to solve the phase problem because of precipitation of heavy metals in such salts. In such a case, the technique of choice would be to transfer the crystal to a carboxylic acid salt

solution, carry out derivatization and finally vitrify. The method is easy and does not involve a multi-stage procedure. Preparation of cryoprotective buffers is based on a simple crystallographic protocol, which is neither time-consuming nor troublesome. The cryoprotection method presented in this paper addresses the current demand for reliable new protocols for cryogenic temperature X-ray diffraction experiments.

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