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Malonate: a versatile cryoprotectant and stabilizing solution for salt-grown macromolecular crystals

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Collection of quality data on high-energy synchrotron sources requires the selection of a cryoprotectant that allows protein crystals to be cryocooled without damage to the crystal and with suppression of ice formation. The use of sodium malonate as a versatile cryoprotectant for salt-grown protein crystals is presented here. In addition to its useful cryoprotectant properties, sodium malonate can also function as a versatile stabilizing solution that allows the manipulation, derivatization and ligand soaking of crystals grown from salt that may not be possible in the crystal mother liquor.

1. Introduction

In the current era of structural genomics and the advent of high-throughput crystallography, most macromolecular crystallographic data are being collected on cryocooled crystals at high-energy synchrotron sources. The selection of a suitable cryoprotectant is an empirical and often time-consuming process in which several compounds, typically salt-free liquids of low freezing point (Petsko, 1975), are tested until a suitable compound is found. Traditionally, crystals grown from polyalcohols are much easier to cryoprotect than crystals grown from salt. Compounds such as MPD, ethylene glycol, glycerol and low-molecular-weight PEGs usually prove to be sufficient cryoprotectants for crystals grown under these conditions. However, crystals grown from high concentrations of salt (*i.e.* ammonium sulfate) can often present a challenge, as these crystals are often intolerant of the addition of the previously mentioned alcohols. Most often, salt-grown crystals are cryocooled in a mixture of their mother liquor with glycerol or are transferred to oil, sometimes with less than satisfactory results.

Solutions of neutral sodium malonate have been shown to be effective precipitants for macromolecular crystallization (McPherson, 2001) and have recently been reported to act as a cryoprotectant (Xing & Xu, 2003). We report here the utilization of 50–100% saturated sodium malonate as a potentially universal cryoprotectant for crystals grown from salt, and present five examples of its utilization in our laboratory. We have found sodium malonate to be a successful cryoprotectant for 100% of the crystals grown from salt under study in our laboratory. In addition, sodium malonate can act as a stabilizing solution for salt-grown crystals, allowing heavy-atom derivatization and ligand-soaking experiments that were not possible in the crystal's native mother liquor.

2. Materials and methods

2.1. Sodium malonate preparation

Saturated neutral sodium malonate was prepared in a similar fashion as has been described previously (McPherson, 2001). Briefly, a quantity of malonic acid suitable to make a final solution of approximately 5 *M* is stirred with a small volume of water. The pH of the resulting solution is adjusted to a final pH of 7.2, initially with solid sodium hydroxide and subsequently by the slow addition of a saturated solution of sodium hydroxide. Caution must be exercised as the process is highly exothermic and titration should be carried out on ice in a fume hood. Dilutions of 20–90% malonate were prepared by dilution of the saturated stock with Milli-Q water (Millipore). Additional sodium malonate was purchased from Hampton Research in the Sodium Malonate Grid Screen. Concentrations greater than 3.0 *M* in this screen were also found to be suitable cryoprotectants.

2.2. Enzyme purification and crystallization

Kex2 from *Saccharomyces cerevisiae* was purified and crystallized from 2.1 *M* ammonium sulfate and 3% DMSO as described previously (Holyoak *et al.*, 2003). Δ^5-3-ketosteroid isomerase (KSI) from *Pseudomonas putida* was purified as described in Kim & Choi (1995) and crystallized from 1.4 *M* ammonium acetate, 0.1 *M* sodium acetate pH 4.6. Xylose isomerase (XyI) from *Bacillus stearothermophilus* was purified and crystallized from 2.1–2.4 *M* ammonium sulfate (T. D. Fenn and D. Ringe, manuscript in preparation). *Aeromonas proteolytica* aminopeptidase (AAP) was purchased from Sigma and crystals were grown from 4.5 *M* NaCl as described previously (Desmarais *et al.*, 2002). Bovine pancreatic γ-chymotrypsin was purchased from Sigma Chemical Company and was

crystallized from 60% saturated ammonium sulfate and 100 m*M* sodium iodide. All crystals were grown by the hanging-drop vapor-diffusion method except for γ-chymotrpsin, which was grown by batch crystallization.

2.3. Cryoprotection and flash-cooling

Except as noted below, crystals were transferred from their mother liquor to a 10 µl drop of sodium malonate in a depression plate with the aid of a Hampton cryoloop. The crystals were allowed to soak for a period of 30 s to 5 min and were subsequently transferred back to the Hampton cryoloop and immersed in liquid nitrogen. The cryocooled crystals were stored under liquid nitrogen until data collection. In the case of Kex2, crystals were unstable on direct transfer into 50% sodium malonate and were first transferred to 40% malonate and then to 50% malonate by the same procedure as outlined above. In the case of KSI, the crystals were found to be stable over the pH range 4–7 present in the Hampton Research Sodium Malonate Grid Screen and were cryocooled over the entire pH range *via* the procedure described above.

3. Results and discussion

3.1. Kex2

The serine protease Kex2 was found to produce diffraction-quality crystals when grown from 2.1 *M* ammonium sulfate and 3% DMSO (Holyoak *et al.*, 2003). Owing to complications arising from the long *c* axis (~370 Å) and the high symmetry of the space group ($P6_522$), the data had to be collected at synchrotron sources on large-area CCD detectors. An exhaustive search of cyroprotectants was undertaken and it was found that the crystals could only be successfully cryocooled in heavy paraffin oil. This resulted in poor cryoprotection, with some diffraction from the residual mother liquor in the loop evident in the images. In addition, data statistics showed poor merging *R* factors ($\geq$20%). The crystals were subsequently found to be stable in 40% malonate indefinitely and could be transferred from 40% sodium malonate to 50% sodium malonate to allow cryocooling. The procedure yielded excellent results, with an overall merging *R* of ~9% (Holyoak *et al.*, 2003). Since Kex2 represented a *de novo* structure determination, experimental phase information was required for structure solution; however, the ammonium sulfate mother liquor was incompatible with many potential heavy-atom compounds (Petsko, 1985). Crystals were derivatized with 5 m*M* K_2OsCl_6, which upon incubation in 40% sodium malonate formed black osmium tetroxide *in situ* and allowed the preparation of a suitable derivative that was used to solve the structure of Kex2 by the SIRAS method (Holyoak *et al.*, 2003).

3.2. *A. proteolytica* aminopeptidase (AAP)

Crystals of AAP grown from 4.5 *M* NaCl have typically been cryoprotected in Paratone N, which allowed the refinement of the structure at atomic resolution (Desmarais *et al.*, 2002). Because the NaCl concentration of the mother liquor is near saturation, it is impossible to soak the relatively insoluble substrates and products of AAP into the crystals to obtain ligand complexes. AAP was found to be stable in 50–60% sodium malonate and when cryocooled diffracted to a similar resolution with similar data statistics as the previously collected data in Paratone N. In addition, the lower degree of saturation of the stabilizing solution allows solubilization of the products and inhibitors of AAP, which has successfully lead to the determination of several product and inhibitor complexes with the enzyme (A. Moulin & D. Ringe, unpublished results).

3.3. Xylose isomerase (XyI)

On a rotating-anode generator at room temperature, crystals of XyI in space group *I*222 diffract to better than 1.4 Å resolution. Based on this observation, it was hoped that synchrotron sources would allow collection of >1 Å data to allow refinement of the structure at true atomic resolution. The crystals were originally cryoprotected in 25% glycerol and flash-cooled in liquid nitrogen. Inspection of the electron-density maps revealed that data collected from crystals cryoprotected in glycerol produced electron-density maps containing disordered regions bordering the solvent channels in the lattice that were well ordered in structures determined from identical crystals at room temperature on a rotating-anode generator (Fig. 1). The crystals were subsequently cryoprotected by soaking in 50% sodium malonate, which eliminated the disorder seen in the glycerol-cryoprotected crystals (Fig. 2) and allowed the determination of a 0.84 Å resolution crystal structure of XyI (T. D. Fenn & D. Ringe, manuscript in preparation).

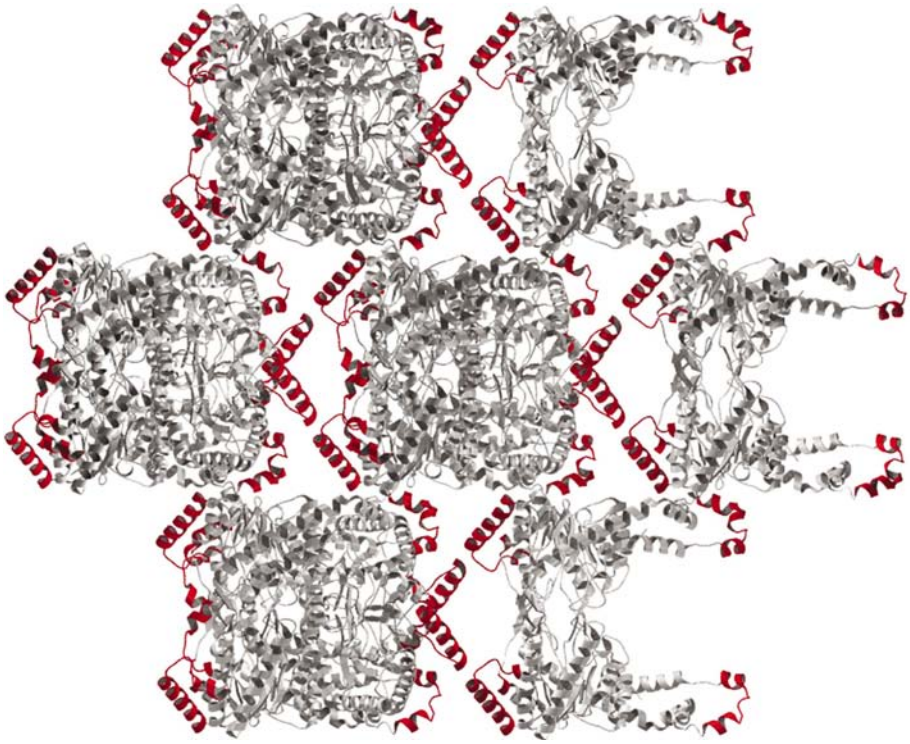

Figure 1
Crystal packing in xylose isomerase. View is along the *c* axis of the $P2_12_12$ lattice. Regions disordered in the glycerol-soaked structures that are not present in the structure determined at room temperature are shown in red and tend to lie in solvent-exposed regions of the lattice.

3.4. Ketosteroid isomerase (KSI)

Crystals of KSI in space group $C222_1$ grow from a mixture of 1.4 *M* ammonium acetate and 0.1 *M* sodium acetate pH 4.6. The crystals were cryoprotected in 50% sodium malonate without prior screening of other compounds. KSI crystals proved to be quite stable under these conditions and data were collected to 1.1 Å resolution at SSRL beamline 11-1. In addition, utilization of the Hampton Research Sodium Malonate Grid Screen demonstrated that the crystals were stable in 3.4 *M* sodium malonate over the pH range 4–7, therefore making structure solution over a wide pH range possible.

3.5. γ-Chymotrypsin

γ-Chymotrypsin crystallizes from highly saturated solutions of ammonium sulfate (60–70%). While crystals could be suitably cryoprotected in 25% glycerol, many problems arise when trying to manipulate crystals or mount them in capillaries, since upon exposure to air the ammonium sulfate immediately begins to precipitate. γ-Chymotrypsin crystals were observed to be stable in 50–60% sodium malonate, which in addition to acting as a suitable cryoprotectant also eliminated problems with crystal handling and manipulation. After growth, the crystals could be transferred to sodium malonate solution for subsequent manipulations.

4. Conclusions

Solutions of sodium malonate have proven to be a useful tool in manipulation and cryoprotection of protein crystals at levels of saturation similar to other cryosalts (Rubinson *et al.*, 2000). In addition to its properties as a cryoprotectant at concentrations of 50% saturation and greater, sodium malonate has proven to be a versatile stabilizing solution for salt-grown protein crystals. It allows derivatization with many heavy-atom compounds, many of which are incompatible with solutions of ammonium sulfate. The typical use of malonate solutions at lower levels of saturation than the mother liquor allows soaks with ligands that may not be possible in the crystal mother liquor. Finally, its pH can easily be matched to that of the mother liquor in the pH range 4–7. In addition, solutions of potassium malonate can be prepared in an identical fashion to sodium malonate solution by substituting potassium hydroxide to adjust the pH of the free acid; the data from the resulting crystals allow easy location of monovalent cation sites owing to potassium's greater electron density and weak anomalous signal.

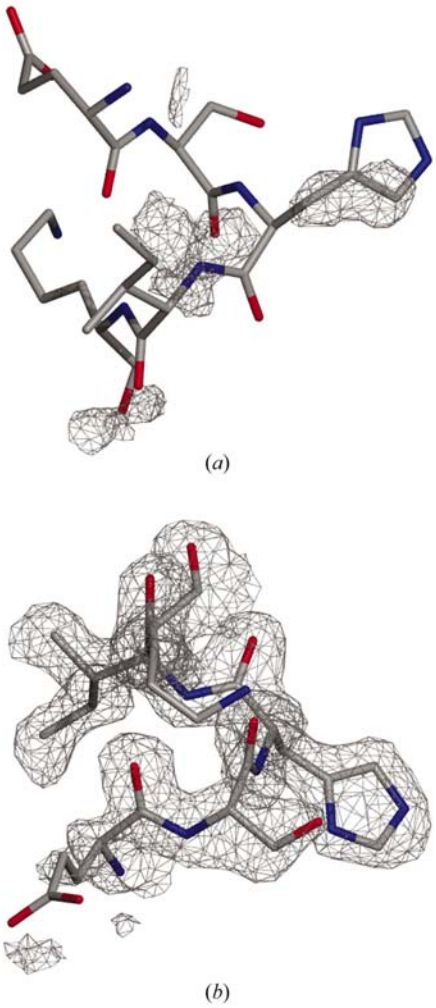

Figure 2
A comparison of the $2F_o - F_c$ electron-density maps contoured at 1σ in the disordered regions. (*a*) 25% glycerol-cryoprotected crystals, (*b*) crystals cryocooled in 50% malonate.

References

Desmarais, W. T., Bienvenue, D. L., Bzymek, K. P., Holz, R. C., Petsko, G. A. & Ringe, D. (2002). *Structure*, **10**, 1063–1072.
Holyoak, T., Wilson, M. A., Fenn, T. D., Kettner, C. A., Petsko, G. A., Fuller, R. S. & Ringe, D. (2003). *Biochemistry*, **42**, 6709–6718.
Kim, S. W. & Choi, K. Y. (1995). *J. Bacteriol.* **177**, 2602–2605.
McPherson, A. (2001). *Protein Sci.* **10**, 418–422.
Petsko, G. A. (1975). *J. Mol. Biol.* **96**, 381–392.
Petsko, G. A. (1985). *Methods Enzymol.* **114**, 147–156.
Rubinson, K. A., Ladner, J. E., Tordova, M. & Gilliland, G. L. (2000). *Acta Cryst.* D**56**, 996–1001.
Xing, Y. & Xu, W. (2003). *Acta Cryst.* D**59**, 1816–1818.