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Ethylammonium nitrate: a protein crystallization reagent

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Ethylammonium nitrate (EAN) is a liquid organic salt that has many potential applications in protein chemistry. Because this solvent has hydrophobic and ionic character as well as the ability to hydrogen bond, it is especially well suited for broad use in protein crystallography. For example, EAN may be used as an additive, a detergent, a precipitating agent or to deliver ligands into protein crystals. A discussion of the crystallization of lysozyme using EAN as a precipitating agent is given here.

1. Introduction

Ethylammonium nitrate (EAN), a low-melting organic salt, is a liquid at room temperature and has a freezing point around 287 K. EAN has water-like characteristics including a capacity for hydrogen bonding and the promotion of micelle formation by some surfactants (Evans *et al.*, 1982). However, EAN is also an organic salt and has ionic character in addition to the hydrophobic character contributed by the ethyl substituent. This unique combination of properties indicates that EAN may be a useful solvent for applications in protein chemistry. Proteins treated with EAN appear to be more stable than those treated with traditional organic solvents. Lysozyme treated with neat EAN and heated to 333 K for 6 h retains 45% activity after such treatment, compared with a 2% retention of activity when treated similarly with DMSO (Flowers, unpublished work). These unique properties make EAN an intriguing solvent and potential crystallization reagent. Here, we demonstrate the utility of EAN as a precipitating agent in protein crystallization.

2. Materials and methods

2.1. Reagents

Ethylamine and lysozyme (L-7773) were purchased from Sigma. Nitric acid, sodium acetate and glacial acetic acid were obtained from JT Baker.

2.2. EAN synthesis

EAN was synthesized from nitric acid and ethylamine in the following manner (Sudgen & Wilkens, 1929). 150 g of 70% nitric acid was added to a 500 ml round-bottom flask. The acid was stirred on ice to allow it to cool. An excess of 70% ethylamine was added dropwise with stirring to the nitric acid. The EAN was stirred overnight with charcoal pellets and then gravity filtered, rinsing the charcoal with methanol to wash off any residual EAN. The product was then lyophilized for one week. The final product was characterized by ^{1}H NMR.

2.3. Crystallization

Hen egg-white lysozyme was dissolved to 50 mg ml^{-1} in 100 m*M* acetate buffer pH 4.5. Hanging-drop crystallization trays were set up with a 500 µl reservoir solution that contained 100 m*M* acetate buffer pH 4.5–5.6 and 300–500 m*M* EAN. The 10 µl hanging drop contained 5 µl of protein solution and 5 µl of reservoir solution. Monoclinic crystals grew at pH 4.5–4.6 over an EAN concentration range of 300–500 m*M*. The tetragonal form grows at pH 5.4–5.6 at an EAN concentration of 300–400 m*M*.

2.4. X-ray characterization

Crystals were mounted in a glass capillary and room-temperature X-ray diffraction data were collected to 1.6 Å resolution on a Rigaku rotating-anode generator equipped with an R-AXIS IV two image-plate detector system. Diffraction intensities were integrated with *DENZO* and scaled using *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Lysozyme crystallized from EAN has two crystal forms. Throughout the pH range studied, large clumps of crystals resembling starbursts were frequently observed. Fortunately, single rectangular crystals (Fig. 1*a*) grew and diffraction data were collected and processed. For the first crystal form, autoindexing using *DENZO* suggested either a

monoclinic or orthorhombic space group; however, orthogonal data processing gave R_{sym} values exceeding 50%. Therefore, these crystals were determined to be monoclinic with unit-cell parameters a = 28.015, b = 62.75, c = 62.883 Å, β = 90.667° (Table 1). Diffraction data along the h axis were not measured, so systematic absences could not be examined. Therefore, the $P2_1$ space group was confirmed by molecular replacement using PDB entry 1b2k. Cycles of rigid-body, positional and B-value refinement with *X-PLOR* (Brünger, 1992) without the addition of water molecules resulted in a final R value of 28.2%. Lysozyme crystallizes in the second crystal form at pH 5.4–5.6 in the tetragonal space group $P4_32_12$ (Fig. 1*b*) with unit-cell parameters $a = b$ = 79.054, c = 38.027 Å (Table 2). This data was also subjected to refinement in *X-PLOR* using PDB entry 1lza and the R value converged at 26.8%. These crystallographic refinements, together with the fact that lysozyme retains activity after treatment with EAN, indicates that lysozyme maintains its native structure in the presence of low levels of EAN and is neither inactivated nor denatured.

Table 1
Monoclinic diffraction data statistics.

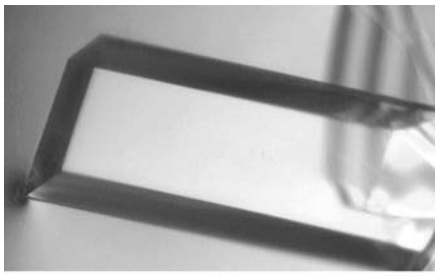
Resolution (Å)	$I/\sigma(I)$	R_{sym}†	Completeness (%)	No. of unique reflections
99–2.91	24.2	0.029	86.7	4086
2.91–2.31	25.0	0.041	95.3	4426
2.31–2.02	20.0	0.052	93.3	4298
2.02–1.83	15.4	0.071	90.7	4200
1.83–1.70	9.2	0.125	89.1	4117
1.70–1.60	5.9	0.195	77.9	3598
All *hkl*	22.5	0.038	88.8	24725

† $R_{sym} = \sum_{hkl}(|\langle I_{hkl}\rangle - I_{hkl}|)/\sum_{hkl} I_{hkl}$.

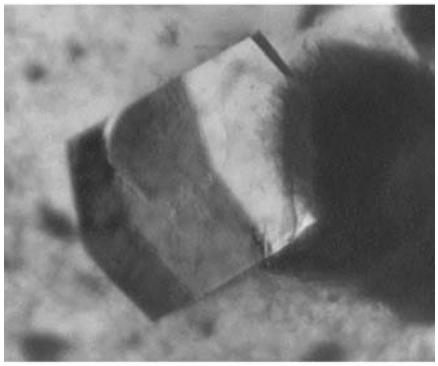
Table 2
Tetragonal diffraction data statistics.

Resolution (Å)	$I/\sigma(I)$	R_{sym}†	Completeness (%)	No. of unique reflections
99–2.91	30.5	0.050	90.7	2653
2.91–2.31	31.8	0.067	100.0	2755
2.31–2.02	26.4	0.080	100.0	2717
2.02–1.83	21.1	0.098	100.0	2691
1.83–1.70	12.9	0.138	100.0	2697
1.70–1.60	7.7	0.202	98.0	2611
All *hkl*	28.5	0.053	98.0	16124

† $R_{sym} = \sum_{hkl}(|\langle I_{hkl}\rangle - I_{hkl}|)/\sum_{hkl} I_{hkl}$.



(*a*)



(*b*)

Figure 1
(*a*) A monoclinic lysozyme crystal grown at pH 4.5 in 100 m*M* acetate buffer using 360 m*M* EAN as the precipitating agent. (*b*) Tetragonal lysozyme grown at pH 5.5 in 100 m*M* acetate buffer using 342 m*M* EAN as the precipitating agent.

EAN has many potential applications in protein crystallography. Hydrophobic ligands, which are generally difficult to introduce into a protein crystal, may dissolve in EAN solutions and be more easily delivered into protein crystals grown with EAN. For example, ferrocene, which is normally insoluble in water, has been shown to be soluble in solutions containing as little as 500 m*M* EAN (Flowers, unpublished work). Some proteins, especially those with exposed hydrophobic regions and that do not respond to traditional precipitating agents, such as peripheral or integral membrane proteins, may have improved solubility in the presence of EAN. Additionally, it can be a useful additive for improving the monodispersity of proteins with multiple aggregation states and increasing crystallizability of the protein solution, as has been shown with other crystallization additives (Gerre-D'Amare & Burley, 1997). For example, the addition of as little as 5% EAN to the protein solution makes previously polydisperse human replication protein A samples monodisperse as determined by dynamic light scattering using a Protein Solutions DynaPro-801 instrument (Borgstahl, unpublished work).

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