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Crystallization of chicken egg white lysozyme from assorted sulfate salts

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Abstract

Chicken egg white lysozyme has been found to crystallize from ammonium, sodium, potassium, rubidium, magnesium, and manganese sulfates at acidic and basic pH, with protein concentrations from 60 to 190 mg/ml. Crystals have also been grown at 4°C in the absence of any other added salts using isoionic lysozyme which was titrated to pH 4.6 with dilute sulfuric acid. Four different crystal forms have been obtained, depending upon the temperature, protein concentration, and precipitating salt employed. Crystals grown at 15°C were generally tetragonal, with space group $P4_32_12$. Crystallization at 20°C typically resulted in the formation of orthorhombic crystals, space group $P2_12_12_1$. The tetragonal ↔ orthorhombic transition appeared to be a function of both the temperature and protein concentration, occurring between 15 and 20°C and between 100 and 125 mg/ml protein concentration. Crystallization from 1.2 M magnesium sulfate at pH 7.8 gave a trigonal crystal, space group $P3_12_1$, $a = b = 87.4$, $c = 73.7$, $\gamma = 120°$, which diffracted to 2.8 Å. Crystallization from ammonium sulfate at pH 4.6, generally at lower temperatures, was also found to result in a monoclinic form, space group C2, $a = 65.6$, $b = 95.0$, $c = 41.2$, $\beta = 119.2°$. A crystal of $\sim 0.2 \times 0.2 \times 0.5$ mm grown from bulk solution diffracted to ~ 3.5 Å.

Keywords: Lysozyme; Sulfates; Trigonal; Monoclinic; Crystallization

1. Introduction

Chicken egg white lysozyme (CEWL) is the most common model protein for macromolecular crystal growth studies. Previous work has shown that anions are the dominant precipitating species for determining CEWL solubility and crystal space group [1]. It has been shown that phosphate, acetate, carbonate, chloride, bromide, citrate, nitrate, iodide, and thiocyanate anions, as well as ethanol and sodium para-toluenesulfonate can crystallize CEWL [1–8].

Sulfates, particularly ammonium sulfate, are one of the most commonly employed precipitants for protein crystallization [9,10]. While the ability to

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grow crystals from sulfate salts was mentioned in the earliest report of lysozyme crystallization [11], native lysozyme was believed to only give amorphous precipitates from sulfate salts. Indeed, the second report on lysozyme crystallization [2] stated that while their "*first crystalline lysozyme preparation was obtained from an acetate buffer on addition of ammonium sulfate*" they had "*never been successful in obtaining crystalline material from a system which contained only the sulfate or the acetate anion.*" This supposed inability to crystallize from sulfate salts has been the basis for several comparative studies of crystal versus amorphous precipitate forming conditions [12–15]. Steinrauf [3] obtained crystals using 10% (0.7 M) sodium sulfate, but in the presence of 1.0 M acetate buffer. Orthorhombic lysozyme crystals were obtained from ammonium sulfate at basic pH, but this was after all lysine side chain amines had been reductively methylated [16]. Riès-Kautt et al. [17] have shown that purified isoionic lysozyme can be crystallized from stoichiometric amounts of sulfate at basic pH. They also reported that crystals could not be obtained at acidic pH. Broide et al. [18] showed that protein straight from the bottle (not further purified) gave tetragonal crystals from magnesium, potassium, and ammonium sulfates at pH 7.8. Vuillard et al. [19] showed that a novel triclinic form of CEWL could be batch crystallized from 75 mg/ml lysozyme, 22% ($\sim$0.9 M) ammonium sulfate at pH 4.6 in the presence of 0.5 M dimethyl ethylammonium propane sulfonate, a zwitterionic solubilizing agent.

We have recently re-examined the use of sulfates as a crystallization agent for CEWL and reported on the sitting drop crystallization of CEWL using ammonium sulfate from pH 4.0 to 7.8 [20]. It was found that high protein and low ammonium sulfate concentrations were key to successful crystallization. Herein, we extend our findings with a report of the crystallization of CEWL from a variety of sulfates over the same pH range, the effects of temperature and protein concentration on the crystal form obtained, the growth of CEWL crystals using isoionic protein titrated to acidic pH using dilute sulfuric acid, and the growth of trigonal and monoclinic crystal forms of CEWL from buffered sulfate salts.

2. Materials and methods

CEWL (Sigma) was repurified by cation exchange chromatography and recrystallized as previously described [21]. The recrystallized protein was then dialyzed against several changes of distilled water, then concentrated using an Amicon YM-3 membrane to the desired final concentration. Completely desalted, or isoionic lysozyme, was prepared by the method of Riès-Kautt et al. [17]. All chemicals were reagent grade or better. All buffers were prepared by making a solution of $\sim$95% final volume containing the desired final molar amount of the buffering species, adding salt to the desired final concentration, then titrating the buffer with the counter ion acid or base to the desired pH. Once the pH was obtained the volume was adjusted to the final value with dH_2O. Crystallizations were performed using the sitting drop technique [22] using a 1 : 1 mixture of protein in distilled water and reservoir solution (10 μl of each solution) for the drop. Several different protein preparations were used over the course of these experiments. Unless otherwise noted the pH values are those of the buffered precipitant (reservoir) solution prior to mixing with the protein. The sitting drops were kept in incubators controlled to the set point $\pm 0.5^{\circ}$. Concentrations refer to those of the starting solution components prior to mixing. In theory, these are also the final values assuming the 1 : 1 mixture undergoes a 50% reduction in volume over the course of the equilibration process.

Suitable crystals were mounted in capillaries for crystallographic analysis. X-rays were produced from a Rigaku rotating anode source operated at 40 kV (70 mA) with a fine 300 μm focus and 300 μm collimator and CuKα radiation at 1.54 Å. Data were collected at room temperature using an *R*-axis II image plate with 105 μm pixel scan. The X-ray diffraction data were processed with the programs Denzo and Scalepack [23].

Solubility measurements were made using the microcolumn method developed in this laboratory [24,25]. Crystals used to pack the columns were prepared by dialysis of an $\sim$150 mg/ml lysozyme solution against 0.1 M sodium phosphate, 0.3 M ammonium sulfate, pH 6.8, in a thermostatted

beaker at 10°C. A portion of the crystals and supernatant solution in the dialysis bag was removed, and the remainder dialyzed against 0.1 M sodium phosphate, 0.4 M ammonium sulfate. About $\frac{1}{2}$ of the crystals and supernatant from this dialysis was removed, and the remainder dialyzed against 0.1 M sodium phosphate, 0.6 M ammonium sulfate. The crystals and supernatant solutions from these successive dialyses were used to pack and equilibrate three sets of columns for solubility determination, with the supernatant solutions from the three dialysis operations being used as stock material for preparation of the respective solubility reservoir solutions.

3. Results

3.1. Crystallization from different sulfate salts

An initial series of sitting drop crystallization trials using a variety of sulfate salts were made at room temperature, which typically varied from ~17 to 24°C. The results from these experiments then led to subsequent experiments in incubators with the temperatures controlled to ± 0.5°C or better. The crystallization conditions tried in the temperature controlled experiments are given in Table 1. Crystals were obtained at every pH and buffer concentration employed with each salt. The differences observed in the results came from variations in the protein and precipitant salt concentrations employed. The results are presented for experiments using three representative pH's, 4.8, 6.8, and 7.8, using sodium acetate, sodium phosphate, and tris-HCl buffers, respectively.

In the initial room temperature experiments, for all sulfates, a general trend was noticed whereby lower protein and precipitant concentrations gave bipyramidal crystals, while higher concentrations gave rod-shaped crystals. The bipyramidal and rod-shaped crystals were identified as tetragonal and orthorhombic forms, respectively. This mixture of crystal forms suggested that the room temperature range spanned the tetragonal ↔ orthorhombic phase change region for these conditions. When better temperature control, at 15 and 20°C, was employed the results shown in Table 2 were obtained. The 15°C sitting drops gave both tetragonal and orthorhombic crystals, while those at 20°C gave orthorhombic.

Table 3 summarizes the diffraction data for the CEWL crystals obtained from the different sulfate salts. Generally, only small crystals were used to collect the orthorhombic data, due to their tendency to form a rather dense mass. Orthorhombic crystals ($\geqslant$0.3–0.4 mm) were somewhat fragile, tended to be cracked, and difficult to separate from other crystals. Manganese sulfate is the only salt which so far has not given tetragonal crystals. Crystallization experiments were not attempted in the pH 6.0 region with manganese sulfate due to the formation of a brown precipitate. Also, both magnesium and manganese sulfates gave crystals to higher (1.2 M and 1.7 M, respectively) salt concentrations than the monovalent cation salts. The unit cell dimensions for the tetragonal crystals ranged from $a = b = 78.6$–78.7 Å, $c = 38.6$ Å, close to the dimensions previously reported for this morphology, $a = b = 78.8$–79.2 Å, $c = 37.9$–38.5 Å [1,3,17, 26–29]. Whether the variations are due to salt-induced alterations in the dimensions or to general variations in the unit cell remains to be determined. The orthorhombic unit cell dimensions for crystals grown from sulfate salts ranged from $a = 30.5$ Å, $b = 56.5$–56.6 Å, $c = 73.5$–73.7 Å, all well within the wider range reported of $a = 30.4$–30.9 Å, $b = 56.0$–59.3 Å, $c = 65.2$–73.8 Å [4,5,26–28,30]. The orthorhombic crystals tended to grow in large numbers. Attempts at reducing the number of crystals nucleated by lowering the protein concentration resulted instead in the formation of tetragonal crystals. Raising the protein concentration to grow more or larger tetragonal crystals resulted in the growth of orthorhombic crystals.

3.2. Crystallization using isoionic protein

Isoionic lysozyme was prepared using a mixed bed ion exchange column by the method of Riès-Kautt et al. [17]. Lyophilized protein prepared in this manner was taken up in distilled water and the pH adjusted to 4.6 by careful titration with 1 M H_2SO_4 and stored at 4°C in microcentrifuge tubes. When this protein was taken out for use in subsequent crystallization trials several were found to

Table 1
Sitting drop crystallization conditions tried. The concentrations given are those at vapor diffusion equilibrium assuming no crystallization has occurred

Precipitant	Buffer	Lysozyme (mg/ml)	Temperature (°C)
0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 M $MnSO_4$	0.1 M Tris, pH 7.8	100	20
0.2, 0.4, 0.6, 0.8 M $MnSO_4$	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	100	20
0.2, 0.4, 0.6, 0.8 M $MnSO_4$	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	100	20
0.3 M $MnSO_4$	0.1 M NaAc, pH 4.8	100, 150	15, 20
0.3 M $MnSO_4$	0.1 M Tris, pH 7.8	100, 150	15, 20
1.0, 1.2, 1.4, 1.6 M $MnSO_4$	0.1 M Tris, pH 7.8	80, 100	15, 20
0.4, 0.6, 0.8, 1.0 M $MnSO_4$	0.1 M NaAc, pH 5.2	80, 100	15, 20
0.2, 0.4, 0.6, 0.8 M $MnSO_4$	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	80	15
0.3, 0.5, 0.7, 0.9 M $MnSO_4$	0.1 M NaAc, pH 4.8	80, 90, 100, 110, 120, 130	10, 15
0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 M $RbSO_4$	0.1 M Tris, pH 7.8	100	20
0.2, 0.4, 0.6, 0.8 M $RbSO_4$	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	100	20
0.2, 0.4, 0.6, 0.8 M $RbSO_4$	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	100	20
0.3 M $RbSO_4$	0.1 M NaAc, pH 4.8	100, 150	15, 20
0.3 M $RbSO_4$	0.1 M NaP, pH 6.8	100, 150	15, 20
0.3 M $RbSO_4$	0.1 M Tris, pH 7.8	100, 150	15, 20
0.3 M $RbSO_4$	0.1 M Tris, pH 7.8	125	15
0.1, 0.2, 0.3, 0.4 M K_2SO_4	0.1 M Tris, pH 7.8	100	20
0.1, 0.2, 0.3, 0.4 M K_2SO_4	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	125	20
0.1, 0.2, 0.3, 0.4 M K_2SO_4	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	125	20
0.3 M K_2SO_4	0.1 M NaAc, pH 4.8	100, 150	15, 20
0.3 M K_2SO_4	0.1 M NaP, pH 6.8	100, 150	15, 20
0.3 M K_2SO_4	0.1 M Tris, pH 7.8	100, 150	15, 20
0.2 M K_2SO_4	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	125	20
0.3, 0.6, 0.9, 1.2 M Na_2SO_4	0.1 M Tris, pH 7.8	100	20
0.3, 0.5, 0.7, 0.9 M $MgSO_4$	0.1 M Tris, pH 7.8	125	20
1.2, 1.4, 1.6, 1.8 M $MgSO_4$	0.1 M Tris, pH 7.8	90	20
0.22, 0.25, 0.27, 0.30 M $MgSO_4$	0.1 M Tris, pH 7.2, 7.4, 7.8, 8.2	125	20
0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 0.6, 0.7 M $MgSO_4$	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	125	20
0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 0.6, 0.7 M $MgSO_4$	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	125	20
0.6, 0.8, 1.0, 1.2 M $MgSO_4$	0.1 M Tris, pH 7.8	90, 100, 125, 150	20
0.3 M $MgSO_4$	0.1 M NaAc, pH 4.8	100, 150	15, 20
0.3 M $MgSO_4$	0.1 M NaP, pH 6.8	100, 150	15, 20
0.3 M $MgSO_4$	0.1 M Tris, pH 7.8	100, 150	15, 20
0.6 M $MgSO_4$	0.1 M NaP, pH 6.8	100, 150	15
0.9 M $MgSO_4$	0.1 M NaP, pH 6.8	100, 150	15
0.5, 0.7, 0.9, 1.2 M $MgSO_4$	0.1 M Tris, pH 7.8	90, 125	15, 20
0.2, 0.3, 0.4, 0.5 M $MgSO_4$	0.1 M NaP, pH 6.8	90, 125	15, 20
0.8, 1.0, 1.2, 1.4 M $MgSO_4$	0.05, 0.1 M Tris, pH 7.6, 7.8, 8.0	60, 70, 80, 90, 100, 110	15, 20, Room temp., 30
1.0 M $MgSO_4$	0.1 M Tris, pH 8.0	70, 80, 90, 100	10
0.3, 0.6, 0.9, 1.2 M $(NH_4)_2SO_4$	0.1 M Tris, pH 7.8	100	20
1.5, 1.8, 2.1, 2.5 M $(NH_4)_2SO_4$	0.1 M Tris, pH 7.8	70	20
0.3 M $(NH_4)_2SO_4$	0.1 M Tris, pH 7.0, 7.2, 7.4, 7.6	100, 150	20
0.3 M $(NH_4)_2SO_4$	0.1 M NaAc, pH 4.0, 4.4, 4.8, 5.2	100, 125, 150	20
0.3 M $(NH_4)_2SO_4$	0.1 M NaP, pH 5.8, 6.0, 6.4, 6.8	100, 125, 150	20

Table 1 (*Continued*)

Precipitant	Buffer	Lysozyme (mg/ml)	Temperature (°C)
0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.5 M $(NH_4)_2SO_4$	0.1 M Tris, pH 7.8	100	42
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.05 M NaAc, pH 4.0, 4.4, 4.8, 5.2	100	20
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.025 M NaAc, pH 4.0, 4.4, 4.8, 5.2	125	20
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.01 M NaAc, pH 4.0, 4.4, 4.8, 5.2	150	20
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.05 M NaP, pH 5.8, 6.0, 6.4, 6.8	100	20
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.025 M NaP, pH 5.8, 6.0, 6.4, 6.8	125	20
0.3, 0.4, 0.5, 0.6 M $(NH_4)_2SO_4$	0.01 M NaP, pH 5.8, 6.0, 6.4, 6.8	150	20
0.3 M $(NH_4)_2SO_4$	0.1 M Tris, pH 7.8	100, 150	15, 20, 30
0.3 M $(NH_4)_2SO_4$	0.1 M NaAc, pH 4.8	100, 150	15, 20
0.3 M $(NH_4)_2SO_4$	0.1 M NaP, pH 6.8	100, 150	15, 20
0.3 M $(NH_4)_2SO_4$	0.1 M NaAc, pH 4.6	40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150	10

Table 2
Lysozyme crystal morphologies obtained as a function of pH, temperature, sulfate salt, and protein concentration

0.3 M precipitant	pH	15°C 100 mg/ml	15°C 150 mg/ml	20°C 100 mg/ml	20°C 150 mg/ml
$(NH_4)_2SO_4$	4.8	t, o	o	o	o
	6.8	t, o	t, o	o	o
	7.8	t, o	t, o	o	o
$MgSO_4$	4.8	o	o	o	o
	6.8	sc	sc, o	sc, o	sc, o
	7.8	o, und	o, und	o	o
$MnSO_4$	4.8	rs, o	rs, o	rs	rs, o
	6.8	–	–	–	–
	7.8	bp	bp, o	bp	bp, o
Rb_2SO_4	4.8	o	o	o	o
	6.8	o	t, o	o	o
	7.8	t, o	t, o	o	o
K_2SO_4	4.8	o	o	o	o
	6.8	t, o	t, o	o	o
	7.8	t, o	t, o	o	o

t = tetragonal.
o = orthorhombic.
sc = salt crystals.
und = undetermined morphology.
rs = round spherulites.
bp = brown precipitate.

Table 3
Crystallographic data for lysozyme crystals grown from various sulfate salts and pH's

Precipitant	Conditions	Space group	Unit cell dimensions	Resolution	Completeness	R-factor
Ammonium sulfate	0.1 M NaAc, pH 4.4 0.3 M $(NH_4)_2SO_4$ Room temperature 100 mg/ml lysozyme	Tetragonal $P4_32_12$	$a = b = 78.71$ $c = 38.60$ $\alpha = \beta = \gamma = 90^\circ$	2.0 Å	99.9%	4.9%
Ammonium sulfate	0.1 M NaAc, pH 4.6 0.3 M $(NH_4)_2SO_4$ 4°C Bulk dialysis	Monoclinic C2	$a = 65.6$ $b = 95.0$ $c = 41.2$ $\alpha = \gamma = 90^\circ$ $\beta = 119.2^\circ$	3.5 Å	70.7%	12.5%
Ammonium sulfate	0.1 M NaAc, pH 4.8 0.3 M $(NH_4)_2SO_4$ Room temperature 125 mg/ml lysozyme	Orthorhombic $P2_12_12_1$	$a = 30.51$ $b = 56.51$ $c = 73.62$ $\alpha = \beta = \gamma = 90^\circ$	1.9 Å	96.3%	4.0%
Magnesium sulfate	0.1 M Tris, pH 7.8 1.2 M $MgSO_4$ Room temperature 90 mg/ml lysozyme	Trigonal $P3_12_1$	$a = b = 87.38$ $c = 73.73$ $\alpha = \beta = 90^\circ$ $\gamma = 120^\circ$	2.8 Å	100%	6.0%
Magnesium sulfate	0.1 M Tris, pH 7.8 0.7 M $MgSO_4$ Room temperature 125 mg/ml lysozyme	Orthorhombic $P2_12_12_1$	$a = 30.49$ $b = 56.52$ $c = 73.63$ $\alpha = \beta = \gamma = 90^\circ$	1.9 Å	92.9%	3.9%
Manganese sulfate	0.1 M NaAc, pH 5.2 0.4 M $MnSO_4$ Room temperature 100 mg/ml lysozyme	Orthorhombic $P2_12_12_1$	$a = 30.53$ $b = 56.60$ $c = 73.50$ $\alpha = \beta = \gamma = 90^\circ$	2.0 Å	81.5%	3.9%
Potassium sulfate	0.1 M Tris, pH 7.8 0.3 M K_2SO_4 15°C 100 mg/ml lysozyme	Tetragonal $P4_32_12$	$a = b = 78.63$ $c = 38.62$ $\alpha = \beta = \gamma = 90^\circ$	1.9 Å	99.9%	7.6%
Potassium sulfate	0.1 M NaAc, pH 4.8 0.1 M K_2SO_4 Room temperature 125 mg/ml lysozyme	Orthorhombic $P2_12_12_1$	$a = 30.53$ $b = 56.48$ $c = 73.60$ $\alpha = \beta = \gamma = 90^\circ$	2.2 Å	95.4%	6.1%
Rubidium sulfate	0.1 M Tris, pH 7.8 0.3 M Rb_2SO_4 15°C 125 mg/ml lysozyme	Tetragonal $P4_32_12$	$a = b = 78.73$ $c = 38.56$ $\alpha = \beta = \gamma = 90^\circ$	2.4 Å	99.0%	6.9%
Rubidium sulfate	0.1 M NaAc, pH 4.0 0.2 M Rb_2SO_4 Room temperature 100 mg/ml lysozyme	Orthorhombic $P2_12_12_1$	$a = 30.50$ $b = 56.53$ $c = 73.70$ $\alpha = \beta = \gamma = 90^\circ$	1.9 Å	94.8%	3.6%

have extensively crystallized. A tube which had a starting concentration of 150 mg/ml lysozyme had produced small tetragonal crystals attached to the sides. Another tube, made to an initial protein concentration of 155 mg/ml, yielded crystals in the "urchin" morphology. The final pH of this solution, estimated with Whatman pH paper (narrow range) was in the range 5.5–5.9, and the final protein concentration was 83 mg/ml. A third tube with an initial concentration of 125 mg/ml gave crystalline shards or flat fragments of indeterminate morphology. A fourth solution starting at 270 mg/ml gave a gel-like precipitate, presumably similar to that observed by Riès-Kautt et al. [17] when they attempted crystallization of isoionic lysozyme adjusted to pH 3.0 by addition of dilute sulfuric acid. In an earlier study Riès-Kautt and Ducruix [1], using commercial protein without further purification, also obtained gels when attempting crystallizations from buffered phosphate and sulfate salts. In this instance they noted that in the case of sodium phosphate after a month small crystals nucleated and then grew within the gel. The phosphate and protein concentrations they employed were higher and lower respectively than those employed for monovalent sulfate salts and protein in this study. In the cases where we clearly observed crystallization in the microcentrifuge tubes we found that they quickly dissolved when brought out at room temperature but could be re-grown when returned to 4°C.

3.3. Trigonal and monoclinic crystal forms

Table 3 includes diffraction data obtained for both the trigonal and monoclinic CEWL crystal forms. Trigonal crystals having a space group of $P3_12_1$ were grown from 0.8–1.2 M magnesium sulfate over the pH range 7.6–8.0, with protein concentrations ranging from 60 to 110 mg/ml (Fig. 1). They grew very slowly, taking 3–4 weeks or longer before even small crystals became apparent. In contrast, 0.7 M magnesium sulfate (pH 7.8) at 125 mg/ml protein in a few days resulted in large numbers of orthorhombic rods while 0.3 M magnesium sulfate (pH 6.8) at 90 mg/ml protein gave tetragonal crystals within a month. When phosphate buffer was used, nicely formed salt crystals, presumably magnesium phosphate, were also observed in the reservoir solution and occasionally in the wells. Subsequent crystallization trials with comparable concentrations of either magnesium bromide or chloride gave only tetragonal crystals. None of the other sulfates tested to date have given trigonal crystals, including manganese sulfate, the only other polyvalent cation tried. At least two months were needed before the trigonal crystals were large enough for X-ray diffraction analysis. Prolonged incubation, six months or more, yielded increased numbers of trigonal crystals, along with a clear granular appearing precipitation.

Initial solubility measurements were attempted using the scintillation method [31]. Neither decreasing nor increasing the temperature reliably produced a change in the photodiode signal indicative of crystal nucleation, suggesting a lack of sensitivity of the solubility on temperature. We then attempted to prepare a mass of crystals by dialysis against 0.3 M ammonium sulfate, 0.025 M sodium acetate, pH 4.6 using a protein concentration of $\sim$130 mg/ml. The dialysis was carried out in a water jacketed beaker, with the circulating refrigerated bath temperature adjusted to 12.5 ± 0.2°C in an attempt to accelerate nucleation. A large crop of small crystals with a truncated triangular cross section was produced after several days (Fig. 2). These crystals were used to seed crystal growth solutions at room temperature. The largest crystals grown from the seeds were ca. 0.25 mm in size. Similar crystals were subsequently obtained after several months in one ammonium sulfate sitting drop experiment (125 mg/ml protein, 0.1 M sodium acetate, pH 4.4, 0.3 M ammonium sulfate) at 20°C. In both cases, X-ray data collection showed the crystals to be monoclinic. In another attempt to produce small crystals for solubility determinations, a dialyzing solution at $\sim$150 mg/ml lysozyme was seeded with tetragonal crystals to induce nucleation at 0.1 M sodium acetate, pH 4.6, 0.3 M ammonium sulfate at 10°C. Small crystals were obtained after several days which could not be visually identified and the solution contents of the dialysis bag were stored at 4°C in a beaker. After one month an additional crop of larger crystals showing similar morphology to Fig. 2 was obtained growing attached to the beaker sides and top

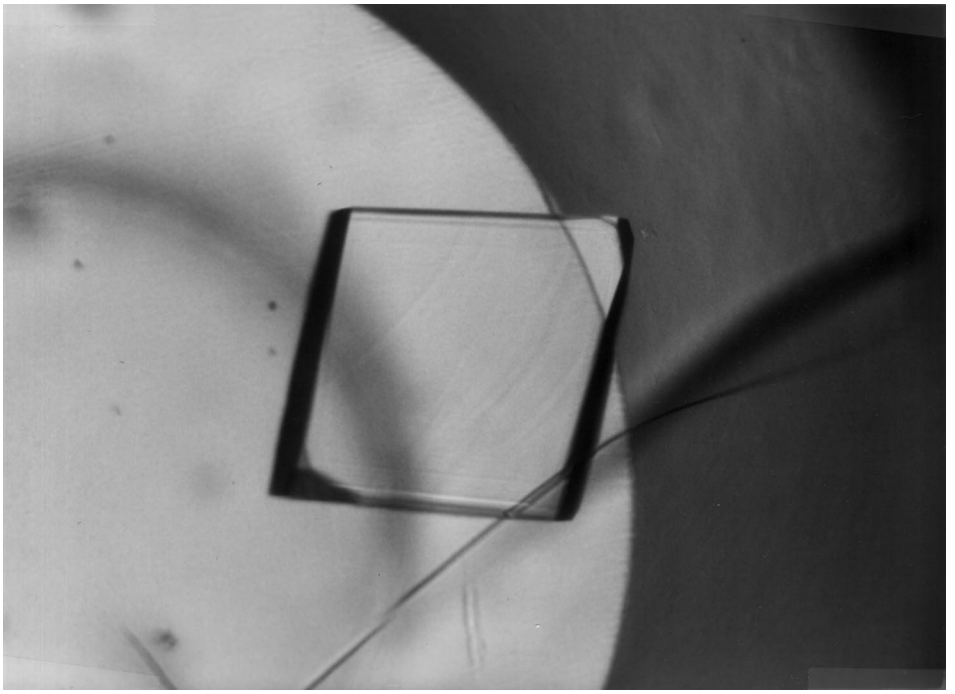

Fig. 1. Trigonal crystal grown from magnesium sulfate.

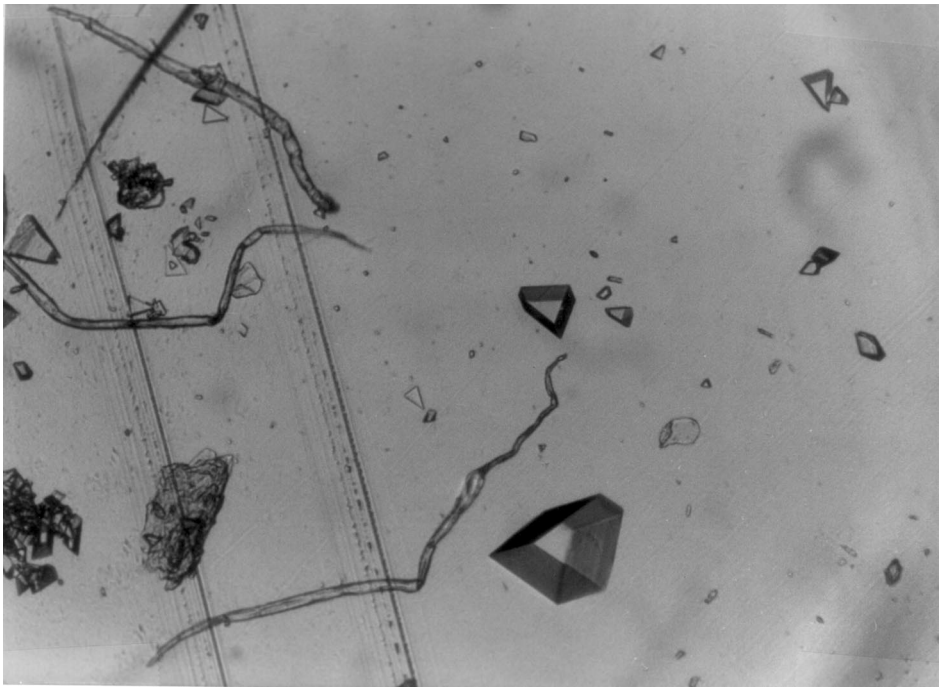

Fig. 2. Monoclinic crystals grown from ammonium sulfate.

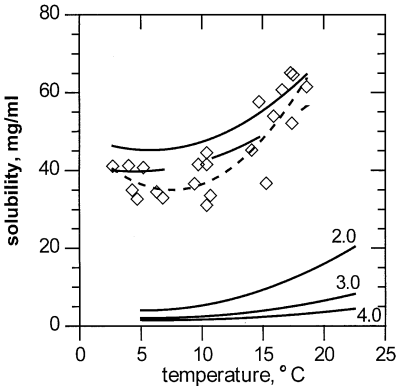

Fig. 3. Solubility curves of crystalline lysozyme obtained at 0.1 M sodium phosphate buffer, pH 6.8. Due to the large scatter and slight variation in the data only every other point is shown for the data collected at 0.3 M ammonium sulfate. The lines shown are second order polynomial fits to the data obtained at 0.3 M (short dashes), 0.4 M (solid line), and 0.6 M (long dashes) ammonium sulfate. Polynomial fits to data obtained for tetragonal lysozyme solubility in 0.1 M sodium phosphate, pH 6.8, at 2.0, 3.0, and 4.0% NaCl is included for comparison.

of the solution. An X-ray data set was collected at 4°C for one ($\sim 0.2 \times 0.2 \times 0.5$ mm) and it was found to be monoclinic, space group C2 (Table 3).

The above results suggested that lysozyme solubility with sulfate is not as temperature dependent as previously found with other anions. Preliminary solubility data obtained at 0.3, 0.4, and 0.6 M ammonium sulfate, 0.1 M sodium phosphate, pH 6.8, is shown in Fig. 3. For comparison, polynomial fits to data previously obtained using 2.0, 3.0, and 4.0% sodium chloride (0.34, 0.51, and 0.68 M respectively), 0.1 M sodium phosphate, pH 6.8 are included on the same graph. The results obtained indicate that under the conditions investigated lysozyme solubility in ammonium sulfate is relatively insensitive to both temperature and salt concentration. However, despite the considerable scatter in the data, solubilities from 0.4 M ammonium sulfate were consistently greater than those from either 0.3 or 0.6 M ammonium sulfate. The ammonium sulfate solubility data had considerable scatter which did not improve after several repetitions. The scatter may in part be due to a crystalline phase change. When the columns were packed the crystals were visually identified as being tetragonal. After the solubility measurements were concluded the apparatus was disassembled and it was noted that the crystal beds were composed of mostly orthorhombic crystals.

4. Discussion

We have previously shown that CEWL can be grown in the presence of ammonium sulfate over a wide pH range [20]. This work extends that finding to other sulfates, and also shows that CEWL can be grown from acidic solution with just sulfate as the counter ion. Consistent with the previous findings, crystallization of lysozyme from sulfate salts requires high protein and low salt concentrations. The exceptions to these appear to be with polyvalent cations. Trigonal lysozyme crystals were only grown at high (0.8–1.2 M) magnesium sulfate concentrations and orthorhombic crystals were grown from manganese sulfate up to 1.6 M, whereas orthorhombic and tetragonal crystals were grown from sulfates concentrations as low as 0.2–0.3 M with the monovalent cations.

Vuillard et al. [19] have also grown lysozyme crystals at acidic pH, in their case in the presence of 0.5 M dimethyl ethylammonium propane sulfonate, a zwitterionic solubilizing agent. The ammonium sulfate concentration used, 22% (~ 0.9 M), was somewhat higher than employed in this work with monovalent cation sulfates. We only tried a few temperature controlled crystallizations at sulfate concentrations $\geqslant 0.7$ M, all of which were at basic pH, as the preliminary room temperature experiments and previous results using just ammonium sulfate [20] had indicated that ~ 0.6 M was the upper limit for monovalent sulfate salts. The differences observed reinforce the suggestion of Vuillard et al. that the dimethyl ethylammonium propane sulfonate moderates protein–protein interactions, serving to prevent or reduce the likelihood of amorphous aggregation. Interestingly, they also found peaks in the electron density map which may be sulfate ions, and one large density region suggesting the presence of a partially ordered detergent molecule. In turn, this suggests that the moderating effects are through interaction of the detergent with a region on the protein which may be responsible for amorphous precipitate formation.

We had previously shown that cation exchange repurified lysozyme which had been dialyzed against distilled water could be crystallized from unbuffered ammonium sulfate without any added buffer [20]. However, the bulk of the crystallizations described in this and the previous work are at relatively low sulfate concentrations in the presence of buffer, and it was subsequently suggested to us that a claim of crystallizing from a particular ion can only be made if one used isoionic protein and only that ion in the crystallizing solution. This result has now been achieved, with isoionic CEWL being crystallized simply by addition of dilute sulfuric acid to adjust the pH to 4.6. If the sulfate ion is not the primary precipitant in this case then the precipitating species must have remained bound to the CEWL through an extensive dialysis against dH_2O followed by passage over a mixed bed ion exchange resin to prepare the isoionic protein, suggesting that it is very tightly bound. Neither the binding nor the crystallographic evidence exist which would support such an argument. The isoionic CEWL-sulfate crystals could be readily dissolved and regrown by cycling them between room temperature and 4°C, respectively, indicating that the solubility is "normal", increases with temperature.

The pH of the isoionic protein solutions was found to have risen as a result of the crystallization process. The pH change is not a surprising result, as the only buffering is from the protein in the solution; sulfate is not a buffering species at this pH. Any process which involves the uptake or release of protons will also result in a change in the pH, especially if the concentration of the buffering species and its counter ion is being reduced by removal into the crystalline state. The increase in pH indicates that in this case protons are removed from the solution by the crystallizing protein. Retailleau et al. [32], in an earlier study on the crystallization of isoionic lysozyme from chloride in the absence or presence of salt, found only a slight increase at acidic pH and a slight decrease at pH 9.0.

Delineation of the CEWL phase change regions for the sulfate salt systems is not as apparent as, for example, crystallization from sodium chloride. The ammonium sulfate data at acidic pH show at least three different crystal forms (monoclinic, tetragonal, and orthorhombic) which can be obtained between 10 and 20°C. The appearance of the orthorhombic form at lower temperatures may be due to the thermal history of the protein, and not the actual nucleation and growth temperature employed ([33], and work in progress this laboratory). However, we have not found a causal relationship for the appearance of the monoclinic form over the tetragonal. Qualitatively we find that at acidic pH the tetragonal form is difficult to nucleate in any quantity in bulk solution or by dialysis. These methods nucleate proportionately large numbers of monoclinic crystals once nucleation is initiated. Conversely, vapor diffusion experiments are more likely to produce a few tetragonal crystals, with only a very few wells at acidic pH ever yielding monoclinic. Overall, on the basis of apparent mass of material removed from solution, it qualitatively appears that at acidic pH the monoclinic form is preferred, by virtue of its lower solubility. The systematic effects of pH, different anions, temperature, or precipitant concentration on the appearance of the monoclinic crystals have not been investigated at this time.

The initial solubility data obtained using the microcolumn method reinforce the empirical observations that lysozyme solubility is not as temperature dependent when crystallizing from sulfate salts as from, for example, chloride salts. This lack of sensitivity of solubility to temperature, along with the apparent phase change which occurred over the course of the measurements, may also explain the considerable scatter in the data. Sazaki et al. [34], using two-beam interferometry, found a progressive increase in their measurement error with a decreasing temperature dependence of solubility for lysozyme, and a similar result has been found for solubility determined by the scintillation method [31]. While there is considerable scatter in the data, two of the three polynomial fits suggest retrograde solubility for lysozyme crystallized from sulfate salts at lower temperatures. We also empirically found, when trying to prepare crystals by temperature controlled dialysis, that our best success in nucleation came around 10°C and not at lower temperatures around 4°C, which further suggests a retrograde solubility region below $\sim$10°C.

The finding that magnesium sulfate can yield trigonal CEWL crystals was particularly interesting. The only prior report of a similar crystal morphology for CEWL is the hexagonal form reported by Haas [5], who grew hexagonal (rhombohedral) crystals of space group $P6_12$ with unit cell dimensions of $a = b = 87.01$ and $c = 73$ Å from a solution of 10% acetone in a bicarbonate solution at pH 8.4, saturated with sodium nitrate. On the other hand, turkey egg white lysozyme, which differs from CEWL by six amino acids, typically crystallizes in the hexagonal form with space group $P6_122$ and unit cell dimensions of $a = b = 70.96$ and $c = 83.01$ Å [35,36].

The growth of the trigonal crystals was very slow and within the first 2–3 months only a few small crystals were produced in a given 24 well sitting drop plate. Increasing the protein concentration did not result in the nucleation of more crystals or faster growth of those obtained. The low number of crystals formed indicates that very little protein was removed from solution, that the solubility is relatively high. In comparison, quite a large amount of protein is removed from solution with crystallization of the orthorhombic or tetragonal forms at lower magnesium sulfate concentrations. This suggests that as one progressively increases the magnesium sulfate concentration a point is reached where the equilibrium solubility begins to rise, that the protein starts to become more soluble. This is supported by the phase partitioning data of Broide et al. [18]. They show, at fixed protein concentration, a decrease in the cloud point temperature with increasing salt concentration for magnesium sulfate at pH 7.8, which would indicate that the solubility is apparently increasing with increasing salt concentration. For both magnesium bromide and chloride their data show that the cloud points first increases (decreasing solubility) with salt concentration, then decreases (increasing solubility), with the highest cloud points appearing around 1.0 and 1.5 M, respectively.

The growth of the monoclinic crystal form is also very slow. In all cases it has appeared only slowly, whereas the tetragonal and orthorhombic forms become apparent within a few days. While the range of conditions for the occurrence of this phase have not been determined, it seems to be favored by lower temperatures. The space group, C2, is different from the more usually obtained $P2_1$ space group for lysozyme [1,3,4,6,19,37–39], making this a second new crystal form for lysozyme obtained from a sulfate salt.

In conclusion, it seems curious that CEWL, which has heretofore seemed so facile in its crystallization behavior over a wide range of conditions, should not be crystallized by sulfate salts. We have now shown that (1) isoionic CEWL can be crystallized from just sulfate ion at acidic pH, (2) CEWL can be crystallized by a variety of inorganic sulfate salts in acidic and neutral buffered solution, and (3) at least two previously undescribed forms of lysozyme can be grown from sulfate salts. The results obtained suggest that previous attempts from buffered solution may have used too high a salt or too low a protein concentration, and that while the presence of buffer ions may facilitate the crystallization process, lysozyme crystals can be obtained at acidic pH using just the sulfate anion.

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