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Crystallization of chicken egg-white lysozyme from ammonium sulfate

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Abstract

Chicken egg-white lysozyme was crystallized from ammonium sulfate over the pH range 4.0–7.8, with protein concentrations from 100 to 150 mg ml^{-1}. Crystals were obtained by vapor-diffusion or batch-crystallization methods. The protein crystallized in two morphologies with an apparent morphology dependence on temperature and protein concentration. In general, tetragonal crystals could be grown by lowering the protein concentration or temperature. Increasing the temperature or protein concentration resulted in the growth of orthorhombic crystals. Representative crystals of each morphology were selected for X-ray analysis. The tetragonal crystals belonged to the $P4_32_12$ space group with crystals grown at pH 4.4 having unit-cell dimensions of $a=b=78.71$, $c=38.6$ Å and diffracting to beyond 2.0 Å. The orthorhombic crystals, grown at pH 4.8, were of space group $P2_12_12$ and had unit-cell dimensions of a = 30.51, b = 56.51 and c = 73.62 Å.

1. Introduction

Ammonium sulfate is one of the most commonly employed precipitants for protein crystallization (Gilliland, 1988). However, the most widely used model protein, chicken egg-white lysozyme (CEWL), has never been successfully crystallized with sulfate ions at neutral or acidic pH. CEWL was believed to produce only an amorphous precipitate from ammonium sulfate, which has been the basis of a number of studies comparing protein–protein and protein–solvent interactions in crystallizing *versus* amorphous precipitating conditions (Kam, Shore & Feher, 1978; Baldwin, Crumley & Carter, 1986; Mikol, Hirsch & Giegé, 1989; Skouri, Munch, Lorber, Giegé & Candau, 1992; George & Wilson, 1994; Ducruix, Guilloteau, Riès-Kautt & Tardieu, 1996). The first successful crystallization of CEWL was with protein, prepared by electrodialysis, from ammonium sulfate in an acetate buffer (Alderton, Ward & Fevold, 1945). However, this could not be repeated (Alderton & Fevold, 1946). Riès-Kautt, Ducruix & Van Dorsselaer (1994) reported that purified isoionic CEWL could be crystallized at basic pH from low concentrations of sulfate. However, they were unable to grow crystals at neutral or acidic pH. Broide, Tominc & Saxowsky (1996) then crystallized CEWL straight from the bottle in the tetragonal form with magnesium, potassium and ammonium sulfates at pH 8.4.

The demonstration that isoionic CEWL could be crystallized from sulfate at stoichiometric ratios at basic pH, followed by its general crystallization from several different sulfates, also at basic pH, led us to reexamine sulfates and CEWL crystallization. Riès-Kautt *et al.* (1994) had indicated an inability to obtain crystals at acidic pH values, and we wanted to determine if there was a critical pH where crystallizability was lost. Instead, we found that crystals could be grown at pH values down to pH 4.0, the lowest limit tested. Herein, we report on the successful crystallization of CEWL from ammonium sulfate over the pH range 4.0–7.8.

2. Materials and methods

CEWL (Sigma) was repurified by cation-exchange chromatography and recrystallized as previously described (Forsythe, Ewing & Pusey, 1994). All chemicals were reagent grade or better. Sitting-drop crystallizations were set up over the pH range 4.0–7.8 (Davies & Segal, 1971), using sodium acetate, sodium phosphate, and tris–HCl buffers at room temperature. Buffer concentrations of 0.1, 0.05, 0.025, and 0.01 *M* were investigated with ammonium sulfate concentrations ranging from 0.3 to 2.5 *M*. Protein concentrations were 100, 125, and 150 mg ml^{-1}. Concentrations refer to the final equilibrium concentration.

Batch crystallizations were performed in 5 ml disposable plastic test tubes at room temperature. Two sets of buffer conditions (sodium acetate, pH 4.6 and sodium phosphate, pH 6.2) were studied. Highly concentrated protein in distilled H_2O was sequentially diluted with 0.1 *M* buffer. Then, 3 *M* ammonium sulfate was slowly added with gentle vortexing to bring the final sulfate concentration to 0.3 *M*. The first tube, with the highest protein concentration, was not diluted with buffer.

Suitable crystals were mounted in capillaries for crystallographic analysis. X-rays were produced with a Rigaku rotating-anode source operated at 40 kV (70 mA) with a fine 300 µm focus and 300 µm collimator and Cu $K\alpha$ radiation at 1.5418 Å. Data was collected at room temperature using an R-AXIS II image plate with 105 µm^2 pixel scan. The X-ray diffraction data were processed with the programs *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993).

3. Results and discussion

In the sitting-drop experiments, crystals appeared at every pH and buffer concentration within 1–14 d. For the batch crystallizations, orthorhombic crystals appeared in all tubes within 1–5 d. At lower buffer concentrations (0.025 and 0.01 *M*), crystals were obtained from 0.4 to 0.6 *M* ammonium sulfate. The 0.1 *M* sodium acetate and sodium phosphate trials were only conducted at 0.3 *M* ammonium sulfate. Crystals were not produced with ammonium sulfate $\geq$0.6 *M*; a phase separation occurred at concentrations $\geq$1.2 *M*; ammonium sulfate concentrations between 0.3 and 0.6 *M* were not tested with 0.1 *M* buffers; and concentrations >0.6 *M* were not explored with buffer concentrations <0.1 *M*.

Crystal morphology varied with the protein concentration and temperature. Those grown from lower protein concentrations (100 mg ml^{-1}) had a tetragonal habit, confirmed by X-ray analysis, although they were somewhat flattened along the fourfold axis. Higher protein concentrations (150 mg ml^{-1}) gave a large mass of chunky rods which were somewhat fragile.

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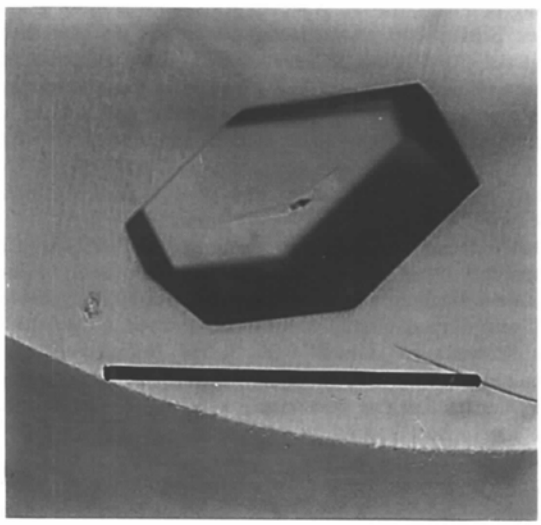
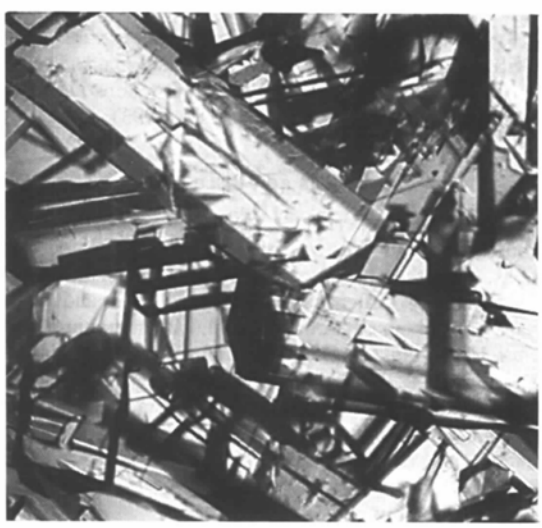
Subsequent X-ray analysis showed them to be orthorhombic, with many being twinned. Fig. 1 is a photomicrograph of these two crystal forms. Isothermal crystallization experiments at 288 and 293 K indicated that tetragonal crystals were favored at 288 K while the higher temperature gave predominantly orthorhombic rods. However, we also found the rods to be favored with higher protein concentrations as well. The tetragonal$\longleftrightarrow$orthorhombic morphology change with temperature is well known (Berthou & Jollès, 1974). However, in this case it also appears to be occurring as a function of the lysozyme concentration. At this point, a pH dependency of the morphology was not clearly evident.

Several crystals were used for X-ray analysis. No quantitative differences were noted as a function of the pH, either for the tetragonal or orthorhombic forms. Table 1 summarizes the crystallographic data obtained. The unit-cell dimensions for tetragonal lysozyme crystals prepared from ammonium sulfate at pH 4.4, $a = b = 78.71$, $c = 38.6$ Å, were slightly outside the ranges previously reported for this morphology, $a = b = 78.83$–79.23, $c = 37.89$–38.5 Å (Palmer, Ballantyne & Galvin, 1948; Steinrauf, 1959; Jollès & Berthou, 1972; Berthou & Jollès, 1974; Riès-Kautt & Ducruix, 1989; Riès-Kautt *et al.*, 1994; Ewing, Forsythe, van der Woerd & Pusey, 1996). Orthorhombic lysozyme unit-cell dimensions vary somewhat more, ranging from $a = 30.4$–30.9, $b = 56.0$–59.3 and $c = 65.2$–73.8 Å (Haas, 1967; Jollès & Berthou, 1972; Berthou & Jollès, 1974; Artymiuk, Blake, Rice & Wilson, 1982; Guilloteau, Riès-Kautt & Ducruix, 1992). However, orthorhombic crystals grown with ammonium sulfate had similar unit-cell sizes within this range, with $a = 30.51$ and 30.75, $b = 56.51$ and 56.32, and $c = 73.62$ and 73.94 Å for crystals grown at acidic and basic pHs, respectively.



(*a*)



(*b*)

Fig. 1. The two crystal forms obtained, in this case by sitting drops at room temperature from 0.1 *M* tris, pH 7.4, 0.3 *M* ammonium sulfate. (*a*) The tetragonal form grown at 100 mg ml^{-1} protein concentration. (*b*) The orthorhombic crystals from 150 mg ml^{-1} protein solutions. The scale bar in (*a*) $\simeq$ 1 mm.

Anions are the dominant precipitating species for determining CEWL solubility and crystal space group (Riès-Kautt & Ducruix, 1989). It has been shown that phosphate, acetate, carbonate, chloride, bromide, citrate, nitrate, iodide and thiocyanate anions, as well as ethanol and sodium paratoluenesulfonate can crystallize CEWL (Alderton & Fevold, 1946; Crick, 1953; Steinrauf, 1959; Haas, 1967; Hogle *et al.*, 1981; Riès-Kautt & Ducruix, 1989; Guilloteau *et al.*, 1992). Steinrauf (1959) obtained fragile monoclinic plates using 10% (0.7 *M*) sodium sulfate, but in the presence of 1.0 *M* acetate buffer. The dominant precipitating anion is not clear in this case.

The possibility remains that the crystallization of lysozyme here was due to the acetate buffer present or some other (unknown) ion rather than sulfate. While this cannot be rigorously ruled out, there are factors which suggest otherwise. First, decreasing the buffer concentration did not result in a loss of crystallizability of the protein. At 0.01 *M* buffer and 150 mg ml^{-1} protein, the ratio of buffer to protein is $\sim$1:1, suggesting that the acetate ion is not a significant factor. Also, batch crystallization experiments with no added buffer still yielded crystals. Second, Jolivatt, Riès-Kautt, Chevallier & Ducruix (1997) have shown that dialysis against 0.1 *M* ammonium sulfate readily displaces chloride ions bound to CEWL. Acetate and phosphate are both weaker precipitants of CEWL than chloride (Riès-Kautt & Ducruix, 1989) and presumably they would also be displaced by sulfate ions. Third, if we assume lysozyme desolubilization is (at least in part) due to protein–precipitant interactions then a rough estimate of the binding equilibrium can be obtained from the precipitant concentrations. Lysozyme is generally precipitated from salt concentrations in the $\sim$0.2–2.0 *M* range, suggesting a very low equilibrium binding constant between the protein and the ions. The postulated presence of a tightly bound anion which remains through a crystallization and dialysis procedure assumes a high binding affinity. Such a tightly bound species should also appear in the crystal structure. The structure of tetragonal lysozyme has now been determined to 1.33 Å (Vaney, Maignan, Riès-Kautt & Ducruix, 1996). Only one Cl^- and one Na^+ have been located in these high-resolution studies. However, no crystallographic structure or solution binding evidence for a high-affinity anion binding site, specifically for acetate or phosphate, has been forthcoming and we conclude that one is not present.

In order to obtain crystals with ammonium sulfate, high protein concentrations are required which suggest that lyso-

Table 1. *Summary of crystallographic data from tetragonal and orthorhombic crystals*

All crystals were grown from 0.3 *M* ammonium sulfate at room temperature. The crystals grown at pH 4.4 and 4.8 were buffered with 0.1 *M* sodium acetate while those at pH 7.4 and 7.8 were buffered with 0.1 *M* Tris–HCl.

Parameter	Crystal			
Space group	Tetragonal $P4_32_12$	Tetragonal $P4_32_12$	Orthorhombic, $P2_12_12$	Orthorhombic $P2_12_12$
Growth pH	4.4	7.8	4.8	7.4
Crystal size (mm)	0.4 × 0.4 × 0.5	0.5 × 0.7 × 0.5	0.7 × 0.3 × 0.3	1.0 × 0.3 × 0.3
Resolution (Å)	2.0	1.9	1.9	1.9
Crystal-to-detector distance (mm)	95.5	82.4	72.2	100.82
Oscillation angle (°)	2	2	2	2
Exposure time (s frame^{-1})	1200	1200	1200	1200
Total oscillation range (°)	200	200	200	84
Number of reflections	59504	59151	75863	9328
R_{sym}* (on I) (%)	4.9	6.9	4.0	6.4
Number of unique reflections	8670	10038	10188	8310
Refined cell (a,b,c)	78.71, 78.71, 38.60	78.63, 78.63, 38.62	30.51, 56.51, 73.62	30.75, 56.32, 73.94
Completeness of data (%)	99.9	99.9	96.3	78.0
$I > 3\sigma(I)$ (%)	90.1	89.7	86.8	75.0

* $R_{sym} = \sum(I_i - \langle I \rangle)/\sum\langle I_i \rangle$ where I_i is the measured intensity of an individual reflection and $\langle I \rangle$ the mean intensity of the symmetry-related measurements of this reflection.

zyme is more soluble in the presence of sulfate ions than chloride ions. This result agrees with the overall trend observed with the anionic lyotropic series and lysozyme solubility (Riès-Kautt & Ducruix, 1989; Pusey & Munson, 1991). Additionally, previous studies on the solubility of lysozyme at varying buffer concentrations provided the impetus to increase the protein concentration at lower buffer concentrations (Forsythe & Pusey, 1996). It is well known that the more chaotropic the anion, the lower the resulting CEWL solubility for a given salt concentration (Riès-Kautt & Ducruix, 1989; Pusey & Munson, 1991). Typically, macromolecular crystallizations use high ammonium sulfate concentrations such as 40–60% saturated ammonium sulfate (100% saturated solution $\simeq 4.05\,M$). Lysozyme crystals were produced at ammonium sulfate concentrations up to 0.6 *M*, with the best results at 0.3 *M*. This suggests that previous attempts were not successful because of a combination of too low a protein and too high an ammonium sulfate concentration. This also suggests that the success of Broide *et al.* (1996) is the result of a phase-partitioning effect, with most of the protein going into one phase and the bulk of the ammonium sulfate going into the other. The protein-rich phase, from which their crystals grew, would then have a more suitable ratio of ammonium sulfate to protein. In light of the above, previous comparative studies of CEWL solution interactions (where it was assumed that ammonium sulfate gave only amorphous precipitates) now need to be re-examined and if necessary the conclusions obtained adjusted.

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