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Crystallization of Previously Desalted Lysozyme in the Presence of Sulfate Ions

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Abstract

Lysozyme, which is known to crystallize readily in the presence of many salts, has never been crystallized by salting out with ammonium sulfate. In the present study, lysozyme was first completely desalted by treatment with strong cation- (H^+ form) and anion- (OH^- form) exchange resins. This leads to a protein solution with only H^+ and OH^- as counterions, corresponding to its isoionic point. Addition of 2.5–3 molar equivalents of H_2SO_4 to isoionic lysozyme decreases the pH value to 9–8 and allows crystallization to take place. The space group was found to be $P4_32_12$, similar to the classical lysozyme crystals grown in the presence of NaCl at pH 4.5, with unit-cell dimensions $a = b = 78.9$, $c = 38.5$ Å. Tentative explanation of the sulfate/lysozyme interaction was addressed by mass spectrometry, and shows non-covalent binding of the ions on the protein.

Introduction

Lysozyme is known to crystallize easily, and has become a standard protein for protein crystal growth studies. This protein has been crystallized under very different conditions, including variations in salt (Steinrauf, 1959; Riès-Kautt & Ducruix, 1989), pH (Alderton & Fevold, 1946; Palmer, 1947; Steinrauf, 1959) and temperature (Jollès & Berthou, 1972; Cacioppo, Munson & Pusey, 1991). Lysozyme crystals belong to different space groups depending on the crystallization conditions (reviewed in Steinrauf, 1959; Riès-Kautt & Ducruix, 1989).

Nevertheless lysozyme is also known to resist crystallization with ammonium sulfate, which is the most commonly used salt for protein crystallization among the crystallizing agents (Gilliland, 1988). Lysozyme crystals have been obtained in presence of 0.77 *M* Na_2SO_4 and 0.5 *M* NaAcO at pH 4.5. They belong to the monoclinic system $P2_1$ ($a = 28.6$, $b = 63.0$, $c = 61.6$ Å, $\beta = 93.5°$, $Z = 4$) (Steinrauf, 1959). Although Na_2SO_4 was present, the high concentration of acetate may play the role of crystallizing agent. In our previous study on solubility determination of lysozyme in presence of various salts (Riès-Kautt & Ducruix, 1989), we never succeeded in crystallizing lysozyme in the presence of sulfate ions, whatever counterions (NH_4^+, K^+, Na^+, Li^+) were present, at 291 K and pH 4.5 (NaAcO 50 m*M*). Increasing the protein or sulfate concentration always led to the formation of a gel.

Light-scattering studies used lysozyme/NaCl and lysozyme/ammonium sulfate systems to exemplify conditions leading to crystallization and to amorphous precipitates, respectively (Kam, Shore & Feher, 1978; Baldwin, Crumley & Carter, 1986; Mikol, Hirsch & Giegé, 1989). So lysozyme/sulfate salt has become an example of a system which does not allow crystallization, but the mechanism is not yet understood.

Studying the effect of additives on solubility, crystallization and more generally on protein–protein interactions in solution, we observed that some crystallizing agents are very effective at crystallizing even at concentrations as low as 0.2 *M* (Riès-Kautt & Ducruix, 1989). On the other hand, we noticed that commercial lysozyme, extensively dialyzed against water, still presented a pH value around 5–6, which implies a presence of at least 9–11 counterions per protein molecule for electrostatic compensation. In order to avoid artefacts due to competition between undetermined concentrations of impurities of an unknown nature and low concentrations of crystallizing agents, we decided to remove all ions present with the protein by desalting it. This is achieved by passing the protein solution through ion-exchange resins, a technique which has already been mentioned by Edsall & Wyman (1958) for the preparation of isoionic protein solutions. They defined the isoionic point of a protein as the pH at which the protein dissolved in water bears zero net charge, with

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only H^+ and OH^- as counterions (Edsall & Wyman, 1958). The isoionic protein can then be brought to the desired pH and/or ionic strength, thus controlling the nature and concentration of the additives to be studied.

For a better understanding of the effect of additives on protein crystal growth, we have studied their interactions in undersaturated lysozyme solutions by different methods (small-angle X-ray scattering, NMR *etc.*) (results to be published). Working with undersaturated solutions allows us to include the sulfate ion in our studies.

In a previous study, Chowdhury, Katta, Beavis & Chait (1990) addressed the problem of the presence of adducts (98 Da) in the spectra of different proteins and peptides, questioning whether it was H_2SO_4 or H_3PO_4, both molecules having an identical molecular weight. Their study also showed that the adduct was *non-covalently* bound to the proteins or peptides. Mass spectrometry appeared to be a suitable approach for testing the isoionic lysozyme, and to compare the spectrum with [lysozyme, $n H_2SO_4$] and [lysozyme, $n H_3PO_4$].

In the present paper we give the mass spectrometry data for isoionic lysozyme, as well as for lysozyme to which H_2SO_4 or H_3PO_4 is added. We also explain the desalting procedure of lysozyme and how finally we were able to obtain lysozyme crystals grown with sulfate ions.

Methods

Desalting of the protein

The salt present in commercial lysozyme may be of a different nature depending on the commercial source, and its amount varies from one batch to another. Treating lysozyme with strong cation- and anion-exchange resins aims to exchange the cations with H^+ and the anions with OH^-. The different steps of the purification are as follows.

(1) *Dissolution of the commercial protein.* The commercial protein is dissolved in pure water (Biosedra) to prepare a solution of about 40 mg ml^{-1}. The pH measured for different batches or commercial sources is around 4.0.

(2) *Dialysis.* The protein solution is dialyzed in a dialysis bag (Spectrapor 7, cut off 6000–8000 Da), three times for 2 h against pure water (Biosedra). The pH of the dialyzed solution reaches about 5.0.

(3) *Cation exchange.* The dialyzed solution is first passed through a cation-exchange resin (15–30 ml resin per g protein). The solution is sucked into a syringe containing the resin (Bio Rad AG 50W-X8 20–50 mesh, H^+ form, reference 142–1421). Contact of the protein solution with the resin is allowed for about 2 min, then the solution is removed from the syringe through a 0.22 μm filter. An equal volume of pure water is sucked into the syringe which is shaken for a few minutes, and the rinsing solution is extracted from the syringe through the 0.22 μm filter. The last step is repeated twice in order to recover the protein. Alternatively, the resin is poured in a small column, with the diameter of the resin bed being about three times larger than the height. The protein solution to be desalted is poured through the column. The pH of the solution becomes more acidic (pH ≃ 3–4), depending on how extensive the dialysis was.

(4) *Anion exchange.* The acidic solution is finally passed very rapidly through the anion-exchange resin (Bio Rad AG 1-X8 20–50 mesh, OH^- form, reference 140–1422, 15–30 ml resin per g protein). This flash chromatography can be performed either by a vacuum system adapted at the bottom of the column, or by nitrogen pressure onto the top of the column. If the protein solution remains in contact with the anion exchange resin too long, slight precipitation (which may be denaturation) is observed. The resin is washed three times with pure water in order to recover the protein. The pH observed for different desalted solutions was 10.7–11.4.

(5) *Freeze drying.* The final solution is deep frozen in test tubes in liquid nitrogen and freeze dried.

The overall yield of the desalting procedure and freeze drying lies between 60 and 90% protein, depending on the rinsing steps. The desalted protein is now called 'isoionic' protein.

The cationic and anionic exchange resins can be regenerated by treating them with 1 *N* HCl and 1 *N* NaOH solutions, respectively (three times the volume of resin), then rinsing with pure water (about 15 times the volume of the resin).

An alternative procedure may be used. The commercial protein is passed directly through a mixed-bed column with both cation-exchange resin (form H^+) and anion-exchange resin (form OH^-) without the previous dialysis step. This procedure is faster and results in smaller volumes to freeze dry. The drawback is that a much larger volume of resin is used (50–100 ml resin per g protein) in order to exchange all the salts (including those which would be eliminated by a dialysis step), and there is no possibility of regenerating the resins, as they are mixed together.

Mass spectrometry

Electrospray mass spectra (Smith, Loo, Edmonds, Baringa & Udseth, 1990) were obtained on a VG BioTech (Fisons) mass spectrometer. This technique takes advantage of the high charge number (z) of a protein undergoing electrospray, the (M/z) ratio to be measured becoming z times smaller than the

molecular weight M. For example, the highest peak of the lysozyme spectrum is 1431.5 Da and corresponds to $[\text{lysozyme} + 10H^+]^{10+}$. The molecular weight M is calculated by multiplying 1431.5 by z (*i.e.* 10) and subtracting $10H^+$; consequently, $M = (1431.5 \times 10) - 10 = 14\,305$ Da. Calibration was performed with horse heart myoglobin. The source parameters were standard for protein analysis (Van Dorsselaer *et al.*, 1990). The samples contain 20 pmol protein μl^{-1} (0.3 mg ml^{-1}). They are prepared by mixing 5 μl protein solution (3 mg ml^{-1}) with 45 μl water/MeOH (1:1).

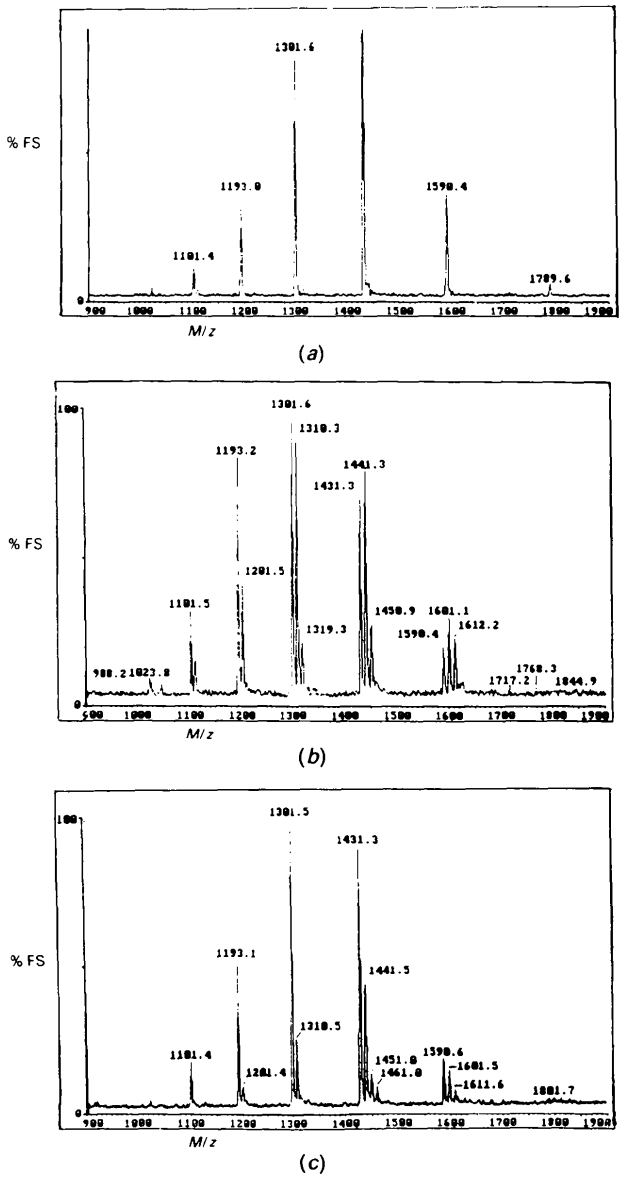

Fig. 1. Electrospray ionization of lysozyme in a mixture of water and methanol (1:1). Concentration = 21 pmol μl^{-1}. (*a*) No acid added, (*b*) 2.5 molar equiv. H_2SO_4 added, (*c*) 9 molar equiv. H_3PO_4 added.

Crystallization and X-ray data

Crystallization is performed by preparing the solution directly under the crystallization conditions (batch method). The required amount of freeze-dried isoionic lysozyme is weighed and water is added for dissolution overnight at room temperature. The solution is then centrifuged for 5 min, and the supernatant filtered over a 0.2 μm filter. The protein concentration is measured by UV optical density at 280 nm, taking an absorbance of 2.66 for a 1 mg ml^{-1} lysozyme solution. Adequate molar equivalents of H_2SO_4 (0.1 N) and then water are added to reach the final concentration or dilution desired.

As isoionic lysozyme is near its isoelectric point (11.1), it dissolves poorly in water ($\leq$85 mg ml^{-1}), compared to lysozyme at pH 4.5 in 50 mM NaOAc where solutions up to $\leq$400 mg ml^{-1} can be prepared. So solutions containing high concentrations of lysozyme ($\geq$100 mg ml^{-1}) are prepared by directly dissolving isoionic freeze-dried lysozyme in dilute H_2SO_4 solution, *e.g.* 21.8 mg of lysozyme in 100 μl 38 mM H_2SO_4. The solution is gently stirred on a Vortex for 2 min, and left to stand for 2 h to allow dissolution. The solution is then centrifuged for 5 min, and the supernatant filtered over a 0.2 μm filter. The protein concentration is measured by UV optical density at 280 nm, taking an absorbance of 2.66 for a 1 mg ml^{-1} lysozyme solution, and the number of molar equivalents of H_2SO_4 *versus* lysozyme are calculated.

The pH is verified with a Metrohm 632 pH meter equipped with a Tacussel MI-410 microelectrode. The crystallization solution is stored in a 0.5 ml Eppendorf test tube, in a thermally regulated chamber at 277 or 291 (0.1) K, depending on the crystallization conditions required.

Crystals have been characterized by X-ray diffraction with a four-circle Philips diffractometer.

Results and concluding remarks

Mass spectrometry

A 20 μM isoionic lysozyme solution (5 μl 3 mg ml^{-1} lysozyme in water, 22.5 μl water and 22.5 μl MeOH) shows a very clean spectrum (Fig. 1*a*). The most intense peak at M/z = 1431.1 Da corresponds to the $(M + 10H^+)^{10+}$, which gives a molecular weight of 14303 (2) Da. In the spectrum of isosionic lysozyme + H_2SO_4 (5 μl lysozyme 2.5 mg ml^{-1} with 5 molar equiv. H_2SO_4 pH 4.4 + 45 μl water/MeOH 1:1) the single peaks of M/z become triplets (Fig. 1*b*). We now observe three peaks at 1431.3, 1441.3 and 1450.9. These peaks correspond to $(M + 10H^+)^{10+}$, $(M + X + 10H^+)^{10+}$ and $(M + 2X + 10H^+)^{10}$, respectively, with X being

an adduct. The mass of X is 98, which corresponds to the molecular weight of H_2SO_4. The relative peak intensities and number of adduct molecules are closely related to experimental conditions, and are not discussed quantitatively, therefore. Recording lysozyme mass spectra in the presence of HCl, AcOH, H_2SO_4 and H_3PO_4, we observed that only sulfuric (Fig. 1*b*) and phosphoric acid molecules (Fig. 1*c*) were adsorbed onto the protein.

These results are an important step towards an understanding of the mechanism of sulfate ions in lysozyme solutions. First, they are correlated with previous observations in crystallization experiments (Riès-Kautt & Ducruix, 1989) where gel formation occurred at acidic pH in presence of sulfate and phosphate salts. Secondly, it is interesting to point out that Chowdhury *et al.* (1990) noticed the presence of additional 98 Da mass with RNase A, RNase S, lysozyme, trypsine, trypsinogen, myoglobin, bradykinin, β-endorphin, dynorphin and a peptide (RRKASGP). It should be noticed that all these molecules have a basic isoelectric point, *i.e.* an excess of basic residues over acidic ones.

Crystallization and X-ray data

At 291 K and pH 7.9, crystallization is observed after 1 week with 218 mg ml^{-1} lysozyme (15.2 mM) and 38 mM H_2SO_4 (2.5 molar equivalents). Large crystals (0.8 mm) have also been obtained at pH 8.8 by macroseeding (Stura & Wilson, 1992) a solution of lysozyme at 140 mg ml^{-1} (9.8 mM) and 28.8 mM H_2SO_4 (2.9 molar equivalents). They show a tetragonal lattice ($P4_32_12$) with $a = b = 78.9$ and $c = 38.5$ Å. This is very similar to the classical form described by Steinrauf (1959) with $a = b = 79.1$ and $c = 37.9$ Å, except for the c axis which is 1.5% larger. The X-ray structure determination of lysozyme/2.5 molar equiv. H_2SO_4 (pH 8.8) is in progress in order to localize the sulfate ion in the electron density of the lysozyme molecule.

At 277 K and pH 8.8, crystals have grown after 1 week with 120 mg ml^{-1} lysozyme (8.4 mM) and 24.4 mM H_2SO_4 (2.9 molar equivalents).

We verified that isoionic lysozyme acidified with H_2SO_4 down to pH 3 (8–9 molar equiv. H_2SO_4) did not crystallize, but gives a gel. This may be explained by non-specific intermolecular bridging of lysozyme molecules occurring either because of a higher amount of sulfate ions or because of the acidic pH. To verify the first hypothesis, we tested the isoionic lysozyme solution brought to pH 8.8 (2.9 molar equivalents H_2SO_4) which did not crystallize unless seeded, and added 6 molar equiv. of Na_2SO_4. The final solution contains 9.2 mM lysozyme (132 mg ml^{-1}), 26.7 mM H_2SO_4 (2.9 molar equiv.) and 55.5 mM Na_2SO_4 (6 molar equiv). Crystals ($P4_32_12$ with $a = b = 78.9$ and $c = 38.5$ Å) up to 1 mm grew spontaneously from this solution. Consequently, the fact that lysozyme does not crystallize in presence of 9 molar equiv. sulfate ions at acidic pH seems to be related to the question of pH rather than the number of molar equivalents.

In crystallogenesis studies, aimed at understanding the influence of the various crystallization parameters, an accurate control of all the parameters is an absolute prerequisite. A preliminary step of protein desalting is helpful for controlling the amount and nature of the additives studied. It also eliminates the risk of competition of the additives with uncontrolled ions already present in the protein solution.

Isoionic lysozyme has been successfully used to address the problem of lysozyme which is unable to crystallize with sulfate ions at acidic pH. Crystals could been obtained in presence of 2.5–3 molar equiv. H_2SO_4 at pH 8 to 9. Formation of gels at acidic pH and mass spectrometry data give a preliminary explanation: the sulfate ions are preferentially non-covalently adsorbed onto the protein. This work is a first step towards understanding the mechanism of interaction between sulfate ions and lysozyme. Different approaches are in progress and should elucidate the mechanism in the near future.

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References

ALDERTON, G. & FEVOLD, H. L. (1946). *J. Biol. Chem.* **164**, 1-5.
BALDWIN, E. T., CRUMLEY, K. V. & CARTER, C. W. JR (1986). *Biophys. J.* **49**, 47–48.
CACIOPPO, E., MUNSON, S. & PUSEY, M. L. (1991). *J. Cryst. Growth*, **110**, 66–71.
CHOWDHURY, S. K., KATTA, V., BEAVIS, R. C. & CHAIT, B. T. (1990). *J. Am. Soc. Mass Spectrom.* **1**, 382–388.
EDSALL, J. T. & WYMAN, J. (1958). *Biophysical Chemistry*, Vol. 1, edited by J. T. EDSALL & J. WYMAN, pp. 600–604. New York: Academic Press.
GILLILAND, G. L. (1988). *J. Cryst. Growth*, **90**, 51–59.
JOLLÈS, P. & BERTHOU, J. (1972). *FEBS Lett.* **23**, 21–23.
KAM, Z., SHORE, H. B. & FEHER, G. (1978). *J. Mol. Biol.* **123**, 538–555.
MIKOL, V., HIRSCH, E. & GIEGÉ, R. (1989). *FEBS Lett.* **258**, 63–66.
PALMER, K. J. (1947). *Struct. Rep.* **11**, 729.
RIÈS-KAUTT, M. M. & DUCRUIX, A. F. (1989). *J. Biol. Chem.* **264**, 745–748.
SMITH, R. D., LOO, J. A., EDMONDS, C. G., BARINGA, C. J. & UDSETH, H. R. (1990). *Anal. Chem.* **62**, 882–899.
STEINRAUF, L. K. (1959). *Acta Cryst.* **12**, 77–79.
STURA, E. A. & WILSON, I. A. (1992). *Crystallization of Nucleic Acids and Proteins: a Practical Approach*, edited by A. DUCRUIX & R. GIEGÉ, pp. 99–126. New York: Oxford Univ. Press.
VAN DORSSELAER, A., BITCH, F., JARVIS, S., GREEN, B., LEPAGE, P., BISCHOFF, R., KOLBE, H. V. J. & ROITSCH, C. (1990). *Biomed. Environ. Mass Spectrom.* **19**, 692–704.