Influence of Chlorides of Mono- and Divalent Metals on the Oligomeric Composition of Lysozyme Crystallization Solutions and Further Crystal Growth

M. A. Marchenkova, P. V. Konarev, A. S. Boikova, K. B. Ilina, Yu. V. Pisarevsky, and M. V. Kovalchuk

Abstract—The influence of the precipitant type (LiCl, NaCl, KCl, NiCl₂, and CuCl₂) on the formation of oligomers (dimers and octamers) in lysozyme crystallization solutions at two protein concentrations has been investigated by small-angle X-ray scattering (SAXS). The same solutions have been used to grow crystals in order to reveal the influence of the oligomeric composition on the crystal growth. The data obtained in this and previous studies on the influence of precipitant concentration yield an inversely proportional dependence of the total content of octamers and dimers on the cation atomic number, which is in agreement with the increase in the ion activity in the lyotropic series for Li⁺, Na⁺, and K⁺ and the increase in the ionic radius for Li⁺, Na⁺, K⁺, Ni²⁺, and Cu²⁺. It is shown that a decrease in the protein concentration in a crystallization solution leads to a decrease in octamer volume fraction at an invariable volume fraction of dimers and reduces the probability of crystal formation.

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INTRODUCTION

Investigation of the spatial structure of proteins inherent in living organisms allows one to gain insight into the mechanisms of functionality of biological molecules in nature.

To date, about 90% of the spatial structures of macromolecules, deposited in the Protein Data Bank (PDB), were determined by X-ray diffraction (XRD) analysis. However, this method is limited by the requirement for single crystallinity of samples. Therefore, studies of the main mechanisms of crystallization of biological macromolecules are of fundamental interest for both the development of crystallography and optimization of the choice of crystallization conditions providing reduced crystal growth time.

In recent years, one could distinguish a transition from the classical crystal-growth scheme to the two-step one, which includes the formation of intermediate cluster precursors. The first such schemes were suggested by the researchers from the Institute of Crystallography of the USSR Academy of Sciences, Professor Sheftal’ in 1957, when analyzing the crystallization from a gas phase, and Grizdeil in 1968, who investigated the crystallization from solutions [1–3]. Active development of this approach has begun in 21st century. To date, the publications in this subject area are rather extensive [4–6]. A particular position is held by a series of studies, performed in the Federal Scientific Research Centre “Crystallography and Photonics” of the Russian Academy of Sciences under the guidance of M.V. Kovalchuk. In those studies, precrystallization cluster precursors in crystallization solutions of several proteins (lysozyme [7, 8], proteinase [9], thermolysine [10], and aminotransferase [11]) and crystallization solutions of inorganic compound of potassium dihydrogen phosphate [12] were experimentally found for the first time using small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS). It was shown for the model protein of lysozyme that, under the growth conditions for lysozyme crystals in a tetragonal system, oligomeric protein particles (dimers and octamers) are formed in the solution [7, 8]; note that octamers are cluster precursors of the crystal. When precipitants (such as LiCl, NaCl, KCl, CoCl₂, NiCl₂, and CuCl₂) were added to the growth of crystals of tetragonal system, are added to the lysozyme solution, the volume fraction of octamers increases in the following orders: K⁺–Na⁺–Li⁺ for monovalent ions and Cu²⁺–Ni³⁺–Co²⁺ for divalent ions [13]. The presence of these lyotropic series (or Hofmeister series, which arrange ions with respect...
to the strength of their effect on various properties (solubility and stability in the case of protein)) is due to their influence on certain parameters of the system under study [14].

This study continues the investigation of the structure of lysozyme solutions under the growth conditions of tetragonal crystals with added NaCl, KCl, LiCl, NiCl₂, or CuCl₂ precipitants, depending on the protein concentration, precipitant type, and temperature, on the P12 EMBL BioSAXS (DESY, Hamburg, Germany) and BM29 BioSAXS (European Synchrotron Radiation Facility (ESRF), Grenoble, France) stations.

EXPERIMENTAL

Materials and Sample Preparation

The samples were fabricated using hen’s egg lysozyme (Sigma-Aldrich, CAS no. 12650-88-3). The mother liquors were prepared using the following inorganic salts: NaCl (CAS no. 7647-14-5, Helicon), KCl (CAS no. 7447-40-7, abcr GmbH), LiCl (technical specifications 6-09-3751-83, Lavenna Stroizinhhiring), CoCl₂ (CAS no. 7791-13-1, Alta Aesar), NiCl₂ (CAS no. 7791-20-0, Alta Aesar), and CuCl₂ (CAS no. 7447-39-4, Acros Organics). All solutions were dissolved in 0.2 M sodium acetate buffer (water resistance 18 MΩ cm). The protein and salts were centrifuged with a rate of 10000 rpm for 10 min. The initial concentration in the protein mother liquor was 80 mg/mL, and the initial concentrations of all salts in the mother liquors were 0.8 and 0.4 M.

SAXS Measurement Technique

Before carrying out SAXS measurements, the lysozyme and salt mother liquors were poured together and mixed in equal volumes. The experiments were performed on the P12 EMBL BioSAXS station of the PETRA III synchrotron radiation source (DESY, Hamburg, Germany) and the BM29 BioSAXS station of the ESRF (Grenoble, France).

Description of the Experiment on the P12 EMBL BioSAXS Station

The energy was 10 keV (λ = 0.124 nm), and a PILATUS 6M 2D detector was used, which makes it possible to record relatively weak scattering signals. The sample-to-detector distance was 3.0 m, and the SAXS data were recorded in the range of backscattering vectors s = 0.02–7.0 nm⁻¹, which corresponds to a resolution of 300–0.9 nm in real space. The SAXS measurements were carried out using a specialized cell for the samples, which consists of a horizontal thermostatted (in the range from 278 to 323 K) quartz capillary with 50-μm-thick walls and a diameter of 1.7 mm, enclosed in a special stainless-steel housing. The exposure time was 50 ms (20 scans were made for each sample measurement). The station was described in more detail in [15]. The sample volume was 40 μL in each measurement. The measurements were performed at a temperature of 20°C.

Description of the Experiment on the BM29 BioSAXS Station

The energy was 12.4 keV, and a PILATUS 1M 2D detector was used. The sample-to-detector distance was 2.9 m. The samples under study were placed in a special thermostatted robotic system in 200-μL polystyrene cells, which were heated simultaneously. The samples were initially heated to 20°C, and then the temperature was reduced to 10°C. Afterwards, the solution was transported (using a robot) from the cell to a continuous-flow quartz capillary 1.8 mm in diameter, which was used in measurements [16]. The solution under study was uniformly transferred through the capillary; in this case, the beam arrived at the same point on the capillary, but each time a new sample region was exposed. Ten scans were made during the sample motion through the capillary. The exposure time was 1 s for each measurement, and beam cross section on the sample was 700 μm². The sample volume was 50 μL in each measurement.

Experimental Data Processing Technique

Averaging of the signal from the buffer solution, subtraction of the averaged buffer signal from the experimental scattering data for the protein solution, and normalization to the protein concentration were performed using the PRIMUS program entering the ATSAS package [17, 18]. As a result, experimental dependences of intensity I on the scattering vector magnitude s (s = 4πsinθ/λ, 2θ is the scattering angle, and λ is the wavelength) were obtained for the protein solutions under different conditions. The angular range was 0.03 < s < 5.0 nm⁻¹. A comparison of successive scans revealed no radiative damage on the samples under study. After the primary treatment, the experimental small-angle scattering curves were processed using the OLIGOMER program [18] to determine the volume fractions of different-order monomers and oligomers. The theoretical curves of oligomeric components were calculated in the CRYSTAL program [19]. The crystallographic structure of lysozyme monomer (PDB ID: 4WLD) was taken as the monomeric component, while the dimer, tetramer, hexamer, and octamer models were obtained according to the technique described in [7]. The approximation quality was estimated by minimizing the residual χ².
between the experimental data and theoretical model approximations from the formula reported in [13].

**Crystallization**

The mother liquors, prepared for SAXS measurements on the P12 EMBL BioSAXS station (DESY, Hamburg, Germany), were also used for crystallization (see Subsection *Materials and Sample Preparation*). The crystallization was performed by the vapor diffusion method in the sessile drop version [20], using the Mosquito-LCP crystallization robot (EMBL, Hamburg, Germany); the drop volume was 200 nL (100 nL of the protein mother liquor + 100 nL of the precipitant mother liquor). The crystals were grown in a ROCK IMAGER automatic visualization system at a temperature of 19°C. The system allows one to observe the protein crystal growth and takes photographs of the drop several times for a long period: on the “zero” day (immediately after loading the crystallization dish) and afterwards on days 1, 3, 7, 14, 28, 56, and 84. The precipitants were the same (NaCl, KCl, LiCl, NiCl₂, and CuCl₂). The final concentrations in the drop were 40 and 20 mg/mL for lysozyme and 0.4 M for the precipitant.

**RESULTS AND DISCUSSION**

*Results of Simulating SAXS Data and Tendencies of the Oligomer Volume Fraction to Change*

In the series of experiments carried out on the P12 (EMBL, Hamburg) and BM29 BioSAXS (ESRF, Grenoble) small-angle scattering stations, the solutions were measured at lysozyme concentrations of 20 and 40 mg/mL and precipitant concentrations of 0.4 and 0.2 M at a temperature of 20°C. The precipitants were inorganic salts: alkaline (NaCl, KCl, and LiCl) and transition-metal (NiCl₂ and CuCl₂) chlorides. Lysozyme solutions without precipitants were measured for comparison. The experimental and theoretical (calculated using the OLLIGOMER program) curves are shown in Fig. 1.

Each combination of the precipitant type and protein concentration was measured several times on the P12 EMBL BioSAXS (DESY) and BM29 BioSAXS (ESRF) stations; the averaged experimental data are listed in Table 1.

The calculated curves of SAXS from oligomeric mixtures for the protein solutions with precipitant are in good agreement with the experimental data in the entire angular range (residual $\chi^2$ does not exceed 1.6, which is indicative of correctness of the proposed processing model). Note that the data obtained in the same experiment at repeated measurements differ only slightly (in some cases, within the processing error).

Analyses of the volume fraction of oligomers in the lysozyme crystallization solutions under identical conditions (protein concentration 20 mg/mL, precipitant concentration 0.4 M), performed on different synchrotron radiation sources, showed that the oligomer volume fractions, averaged over several measurements, differ in the relative ranges of 1–15% (the largest difference of 20% for the LiCl precipitant) for octamers and 25–30% for dimers. In addition, when performing repeated experiments on the same station, the volume fractions could differ under certain conditions by 1.2–1.5% (on the absolute scale) for dimers.
and 0.3–0.5% for octamers; with allowance for this ambiguity, we can suggest that the results presented in Table 3 (measured under different conditions) are consistent. However, the volume fractions of tetramers and hexamers are 0% in all the experiments. The difference between the values obtained on different stations and in repeated experiments in one series may be due to the following factors: inevitable discrepancies in the preparation protocol (time interval between the precipitant addition and actual measurement), different levels of beam attenuation (aimed at excluding radiative damage of the sample), initially different incident-beam intensities, different degrees of purity of the buffer solution (and the presence of impurities), beam stability on the experimental stations, and efficiency of robotic filling of the samples.

On average, octamers demonstrate more stable behavior (their volume fractions obtained in different series of experiments on different sources change only slightly, in comparison with the dimer contents), while dimers to all appearances are less stable formations, and their percentages may change significantly in different experiments.

In all the measurements (except for rows 2 and 8), the dimer volume fraction increases for the used precipitants arranged in the following order: \( \text{Cu}^{2+}, \text{Ni}^{2+}, \text{K}^+, \text{Na}^+, \text{Li}^+ \).

The volume fraction of dimers and octamers increases for the same order of ions as in the case of dimers (\( \text{Cu}^{2+}, \text{Ni}^{2+}, \text{K}^+, \text{Na}^+, \text{Li}^+ \)) or decreases in the opposite order (\( \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Ni}^{2+}, \text{Cu}^{2+} \)), which is consistent with the increase in the ion activity in the Hofmeister (lyotropic) series for \( \text{Li}^+ \), \( \text{Na}^+ \), \( \text{K}^+ \) and the increase in the ionic radius for \( \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Ni}^{2+}, \text{Cu}^{2+} \).

**SAXS Measurements in Time**

For the crystallization conditions with the protein concentration of 20 mg/mL and the precipitant (LiCl, NaCl, KCl, NiCl\(_2\)) concentration of 0.43 M, the studies were performed on the BM29 station (ESRF, Grenoble, France) at instants of 0 min (here, this value actually indicates the time of measurement onset rather than mixing; the measurement onset was delayed with respect to mixing of the protein solution and the precipitant solution by ~10–15 min), 100 min, and 170 min at a temperature of 20°C. The results of processing the experimental data are listed in Table 3.

In the course of time (from 0 to 170 min) after the mixing, the octamer and dimer contents in the solution change only slightly. This allows one to exclude the contribution of the time interval between precipitant addition to protein and actual measurement of the solution from significant factors that affect the dispersion between oligomer contents obtained in different experiments.
Crystal Growth under the Investigated Conditions

The crystals were grown from the same mother liquors that were prepared for SAXS analysis on the P12 station (EMBL, Hamburg, Germany). Crystallization in three drops was stated for each precipitant type. The numbers of experiments, in which lysozyme crystals were formed, are given in the figure captions, and the SAXS data on the average octamer volume fractions in solutions are reported.

Photographs of the crystals grown with protein concentrations of 40 and 20 mg/mL are given in Table 4. The photographs were taken after 56 days (except for the photographs of the crystals with KCl and CuCl₂ precipitants, which were taken on day 84). All presented crystals grew on the next day, except for the crystals in the solutions with the KCl and CuCl₂ precipitants (protein concentration 20 mg/mL), which grew after 84 days.

At the lysozyme concentration of 40 mg/mL in a drop, the crystals grew in all three drops at the octamer volume fraction in the solution ranging from 4.05 to 6.70% (the largest and smallest values were observed with the LiCl and CuCl₂ precipitants, respectively). A decrease in the protein concentration from 40 to 20 mg/mL (which also leads to a decrease in the octamer volume fraction at an invariable volume fraction of dimers in the solution) reduces the probability of formation of crystals (they are completely absent in the case of...
CuCl₂ precipitant (1.95% octamers)). When using other precipitants, the presence of octamers in the solution (at their average volume fraction of 2.15% or higher) correlates with the fact that the growth of lysozyme crystals was observed in these solutions.

**CONCLUSIONS**

The oligomeric composition of lysozyme crystallization solutions with different precipitants (NaCl, KCl, LiCl, NiCl₂, and CuCl₂) was determined by the small-angle X-ray scattering method on the P12 EMBL BioSAXS (DESY, Hamburg, Germany) and BM29 BioSAXS (ESRF, Grenoble, France) stations. It was shown for different protein and precipitant concentrations and for different temperatures that the solutions contain only dimers and octamers; the previously revealed tendencies of the volume fraction of octamers to increase with increasing supersaturation were also confirmed [13].

A twofold decrease in the protein concentration in the crystallization solution, which leads to a decrease in the octamer volume fraction at an invariable dimer volume fraction, reduces the probability of formation of crystals (they are completely absent in the case of the CuCl₂ precipitant (1.95% octamers)). When using other precipitants, the presence of octamers in a solution (at their average volume fractions of 2.15% or higher) correlates with the observation of the growth of lysozyme crystals in these solutions. Thus, it was shown that the formation of the precrystallization oligomeric phase in the initial stage of crystallization in a solution may be one of necessary conditions for the growth of a protein crystal in solution.

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