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Investigation of the protein pre-crystallization solution using analytical ultracentrifugation

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Analytical ultracentrifugation was used to study the crystal growth units in hen egg-white lysozyme pre-crystallization solution. Solutions containing various concentrations of lysozyme and NaCl in 50 m*M* sodium acetate buffer were used for experiments. The crystallization solution was ultracentrifuged using a mode where the sedimentation and diffusion are in equilibrium. The protein concentration gradient in the centrifugation cell was measured by light absorption and the molecular weight was calculated from the concentration gradient data. The results were analyzed assuming that the molecules have no interaction with each other. In all solutions except for 0.4 *M* NaCl, 30 mg ml^{-1} lysozyme solution, it was shown that the molecular weight falls in the range 12 000–16 500 Da. In 0.4 *M* NaCl, 30 mg ml^{-1} lysozyme solution no analysis was made because crystals appeared at the bottom of the cell after centrifugation. Since the calculated molecular weight of lysozyme monomer is 14 400 Da, it was concluded that the lysozyme molecule predominantly exists as a monomer in undersaturated and supersaturated solutions.

1. Introduction

A variety of methods, such as dynamic light scattering (Mikol *et al.*, 1989; Azuma *et al.*, 1989; Georgalis *et al.*, 1995; Schaper *et al.*, 1997; Kuehner *et al.*, 1997; Tanaka *et al.*, 1999) static light scattering (Muschol & Rosenberger, 1995, 1996), atomic force microscopy (Li *et al.*, 1999) small-angle neutron scattering (Boué *et al.*, 1993; Niimura *et al.*, 1995, 1999), small-angle X-ray scattering (Finet *et al.*, 1998) and other methods (Wilson & Pusey, 1992; Miyashita *et al.*, 1994; Wang *et al.*, 1996; Michinomae *et al.*, 1999), have been used to investigate the growth units of protein crystals, mostly using lysozyme as a model protein (summarized in Table 1). However, their results are not completely consistent, with the growth unit resulting in monomer, dimer, tetramer or larger units.

We have investigated the same problem using the analytical centrifugation equilibrium mode to identify the crystal growth units in hen egg-white lysozyme pre-crystallization solution. Analytical centrifugation equilibrium mode and sedimentation velocity mode has long been used to determine molecular weight, sample purity, conformational changes and to study the molecular interactions (for example, Schachmann, 1959; Tanford, 1961; Sophianopoulos & Van Holde, 1961, 1964; Sophianopoulos *et al.*, 1962; Fujita, 1975; Behlke & Knespel, 1996; Behlke & Ristau, 1999; Solovyova *et al.*, 2001). However, no studies have been performed using this method with an aim to identify crystal growth units in lysozyme crystallization solutions. The principles of equilibrium ultracentrifugation are as follows (Tanford, 1961). The sample solution in the cell is ultracentrifuged in a low velocity until the solute sedimentation and diffusion is in equilibrium. Since the system is in equilibrium, the free energy difference of the centrifugal force ($\Delta G_{\rm centri}$) at two positions in the cell (X_A and X_B) is equal to that of the diffusive force ($\Delta G_{\rm diff}$) at these points.

$$\Delta G_{\rm centri} = -\int_{X_A}^{X_B} m' X \omega^2 \, {\rm d}X, \qquad (1)$$

$$\Delta G_{\rm diff} = kT \ln \frac{C_B}{C_A}. \qquad (2)$$

Table 1
Brief summary of the past research.

Experimental method	Results	Reference
Dynamic light scattering	Hydrodynamic radius increased from 2.2 nm to 3.2 nm and decreased back to 2.2 nm during crystallization. Growth unit may be either free-lysozyme molecules or small aggregates.	Mikol *et al.* (1989)
	21–25 nm size clustering was detected. The protein concentration gradient around the crystal was also measured by interferometry. Taking into account the crystal growth rate reported in the past, growth units are monomers.	Azuma *et al.* (1989)
	Particles 12 to 15 times larger than monomeric lysozyme were observed by DLS and X-ray small angle scattering. The transition of monomer to dimer was also observed.	Georgalis *et al.* (1995)
	12–35 nm size clusters were observed by DLS and scanning force microscope. The building unit of the fractals is not the lysozyme monomer but nanometre-sized protein aggregates.	Schaper *et al.* (1997)
	Lysozyme does not aggregate significantly as ionic strength increases, even at salt concentrations near the point of salting out precipitation.	Kuehner *et al.* (1997)
	The change from monomer to unit (few molecules), unit to clusters (100 nm to 1 µm) was observed in supersaturated solution. In undersaturated solution, the molecules were monomolecularly dispersed.	Tanaka *et al.* (1999)
Static light scattering	Prior to nucleation, the intrinsic cluster formation cannot be distinguished from the scattering background of the monomers and its interaction-induced changes.	Muschol & Rosenberger (1995, 1996)
Small angle neutron scattering	Undersaturated solution is a mixture of monomer and dimer. There are dimers, tetramers, octamers in the supersaturated solution, and as the supersaturation increases, the aggregate size increases. The growth units are larger than dimers and probably correspond to tetramers or octamers.	Boué *et al.* (1993)
	Aggregates are found in undersaturated solution, and the size of aggregates increases with the salt concentration. In supersaturated solution, two kinds of aggregates were found (type I, 20–60 nm; type II, 2.5–4 nm).	Niimura *et al.* (1995, 1999)
Small angle X-ray scattering	No indication of oligomer formation was found in supersaturated solution. Lysozyme crystals in NaCl grow by addition of monomeric particles.	Finet *et al.* (1998)
Interferometry	The diffusion coefficient was estimated from the concentration distribution data around the crystal. The hydrodynamic Stokes radius coincided with lysozyme monomer.	Miyashita *et al.* (1994)
Cross-linking	Glutaraldehyde cross-linking followed by SDS–PAGE was used to detect aggregates. The aggregates increased by addition of single units (monomer to dimer, dimer to trimer) as the supersaturation increased. No aggregates were detected in undersaturated solution.	Wang *et al.* (1996)
Electron microscopy	Short thread-like structures and 15 nm size spherical structures were found in supersaturated solution sample prepared for electron microscopy. The spherical structure may be growth units.	Michinomae *et al.* (1999)
Atomic force microscope	The (110) face step is bimolecular height, and the step proceeds with the addition of 4_3 helix (tetramer). Growth units are tetramers.	Li *et al.* (1999)
Dialysis	Aggregates existed in under- and super-saturated solution. (110) face growth rate data supports the theory that lysozyme crystals grow by addition of preformed aggregates and not by monomer addition.	Wilson & Pusey (1992)

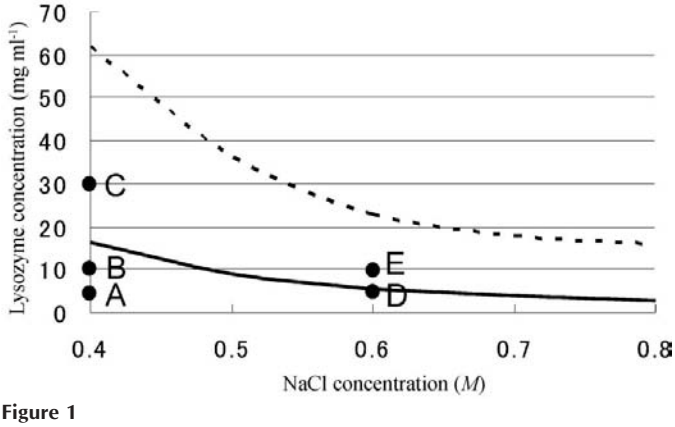

Figure 1
Lysozyme phase diagram. The solid line represents the solubility curve and the dashed line represents the 72 h crystallization line. A–E indicates the experimental conditions.

Here, m' is buoyancy-corrected mass and ω is the angular velocity, k is the Boltzmann constant, T is the temperature, C_A is the solute concentration at X_A and C_B is the solute concentration at X_B. Since the solute concentration gradient in the centrifugation cell is measured by light absorption, the actual molecular mass m is calculated from (3),

$$m' = m(1 - \bar{\nu}\rho) \qquad (3)$$

where $\bar{\nu}$ is the partial specific volume and ρ is the solvent density.

The advantage of the analytical ultracentrifugation equilibrium mode is that the molecular weight of the component species of the solution can be calculated on the basis of the mass irrespective of the shape. An interacting system can be considered by using the second virial coefficient and association/dissociation constant in the analysis. We report here the first application of this method for the study of crystal growth units in protein crystallization solutions.

2. Materials and methods

A Beckman XL-I was used for ultracentrifugation and the equilibrium mode was selected. The reason we used the equilibrium mode is that it is more suited for accurate determination of the molecular weight than the sedimentation mode. The reproducibility was checked by carrying out multiple experiments. The centrifuging velocity was in the range 12 000–18 000 rev min^{-1} and the centrifuging time was in the range 20–70 h. We checked the concentration profile change in the cell with time, to make sure that the system was in equilibrium. The temperature was controlled at 293 K.

The protein concentration was measured by the absorbance at 310 nm. The reason this wavelength was used is that at 280 nm the absorbance is very strong and too intense for quantitative measurement. The linear correlation between the lysozyme concentration and the absorbance at 310 nm was verified prior to experiments.

Analysis was carried out using the Beckman software XL-A based on (1)–(3). The solvent density and the protein partial specific volume $\bar{\nu}$ value, 1.017 g cm^{-3} and 0.72 cm^3 g^{-1}, respectively, were determined in the present study. The results were analyzed assuming that the molecules have no interaction with each other and the virial coefficient was set to zero.

Lysozyme was purchased from Seikagaku Kogyo. NaCl and 50 m*M* sodium acetate buffer pH 4.5 was used for the pre-crystallization solution. The lysozyme phase diagram at 293 K with the 72 h crystallization line was made by microbatch experiments. This was necessary because crystallization should not occur in the centrifugation cell during the experiment in order to keep the system in equilibrium. Five experimental conditions were selected to obtain results with under- and supersaturated solutions (Fig. 1). These solutions were subjected to ultracentrifugation experiments for detection of existing molecular species.

3. Results and discussions

Fig. 2 shows an example of the experimental absorbance data across the centrifugation cell. The dashed line represents the raw data and the solid line represents a valid part of the line used for analysis. A gradient is formed with the concentration continuously changing along the radius of the cell.

In all solutions except for condition C, it was shown that the molecular weight is in the range 12 000–16 500 Da (Table 2). From these data and the calculated molecular weight of lysozyme monomer of 14 400 Da, we may conclude that the lysozyme molecules predominantly exist as a monomer either in undersaturated and supersaturated solutions.

In solution C, no analysis has been made because crystals appeared at the bottom of the cell after centrifugation. The absorption data were out of range at the cell bottom, perhaps owing to intense light scattering by the crystals.

Though it was found that monomers are dominant in the pre-crystallization solution, we could not exclude the possibility that an undetectable number of dimers or tetramers or larger units existed in the solution. However, from the present analysis, it is reasonable to consider that monomers are most probably the growth units. This result is consistent with the previously published results that lysozyme crystals grow by addition of monomeric particles. For example, Finet *et al.* (1998) performed small-angle X-ray scattering (SAXS) experiments with lysozyme crystallization solution and showed that lysozyme molecules were present in solution as monomers. They did not find any indication of oligomer formation. Even in the presence of crystals, the interactions between the lysozyme in solution corresponded to monomeric particles interacting through a short-range attractive potential. Muschol & Rosenberger (1995, 1996) also reported from their static and dynamic light-scattering experiments that the intrinsic cluster formation cannot be distinguished from the scattering background of the monomers and its interaction-induced changes. Miyashita *et al.* (1994) used interferometry to visualize the protein concentration around the growing crystal. The diffusion coefficient of the unit was estimated from the concentration distribution data. It was shown that the hydrodynamic Stokes radius coincided with lysozyme monomer, which means the growth unit is a monomer.

Behlke & Knespel (1996) carried out sedimentation velocity experiments to analyse the nucleation process in a relatively high supersaturation (estimated $\sigma = 5.7$ with the solubility calculated from the data of Cacippo & Pusey, 1991). They reported that precrystallization aggregates existed in solution as well as a predominance of monomers. Our present study did not show the sign of formation of pre-nucleus within the time of experiment. This is owing to the low supersaturation, because nucleation time is a function of supersaturation. According to our phase diagram, solution E is a less supersaturated solution [$\sigma = (C - \mathrm{Ce})/\mathrm{Ce} = 1.16$] and is metastable in 72 h, so it is probable that no pre-nucleus was formed.

In conclusion, analytical ultracentrifugation could be used as a promising and complementary method of light scattering and other methods to study the crystal growth units of protein crystallization solution.

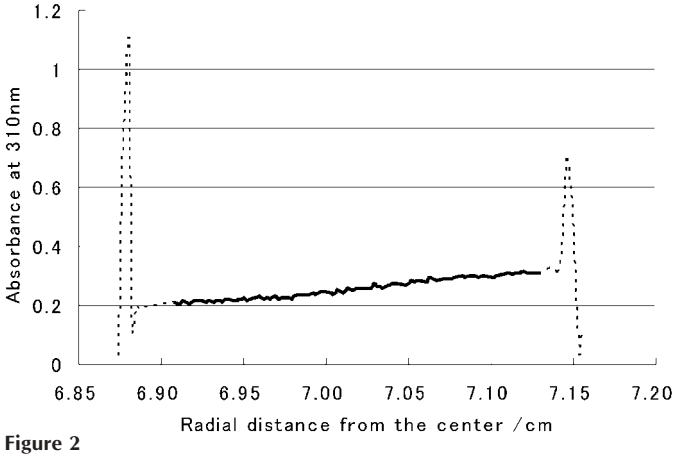

Figure 2
An example of the absorbance data across the centrifugation cell. The dashed line represents the raw data and the solid line represents the data used for analysis.

Table 2
Calculated molecular weight under each experimental condition.

Experimental condition	Concentrations	Solution (supersaturation†)	Calculated molecular weight (g mol^{-1})
A	0.4 *M* NaCl, 5 mg ml^{-1} lysozyme	Undersaturated ($\sigma = -0.63$)	14300 ± 2000
B	0.4 *M* NaCl, 10 mg ml^{-1} lysozyme	Undersaturated ($\sigma = -0.25$)	14300 ± 1000
C	0.4 *M* NaCl, 30 mg ml^{-1} lysozyme	Supersaturated (crystals) ($\sigma = 1.24$)	No analysis
D	0.6 *M* NaCl, 5 mg ml^{-1} lysozyme	Around solubility ($\sigma = -0.08$)	14300 ± 2000
E	0.6 *M* NaCl, 10 mg ml^{-1} lysozyme	Supersaturated ($\sigma = 1.16$)	14300 ± 2000

† Supersaturation σ was calculated as $\sigma = (C - \mathrm{Ce})/\mathrm{Ce}$, where Ce is the solubility and C is the protein concentration.

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