Two types of amorphous protein particles facilitate crystal nucleation

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Nucleation, the primary step in crystallization, dictates the number of crystals, the distribution of their sizes, the polymorph selection, and other crucial properties of the crystal population. We used time-resolved liquid-cell transmission electron microscopy (TEM) to perform an in situ examination of the nucleation of lysozyme crystals. Our TEM images revealed that mesoscopic clusters, which are similar to those previously assumed to consist of a dense liquid and serve as nucleation precursors, are actually amorphous solid particles (ASPs) and act only as heterogeneous nucleation sites. Crystalline phases never form inside them. We demonstrate that a crystal appears within a noncrystalline particle assembling lysozyme on an ASP or a container wall, highlighting the role of heterogeneous nucleation. These findings represent a significant departure from the existing formulation of the two-step nucleation mechanism while reaffirming the role of noncrystalline particles. The insights gained may have significant implications in areas that rely on the production of protein crystals, such as structural biology, pharmacy, and biophysics, and for the fundamental understanding of crystallization mechanisms.

nucleation | protein | lysozyme | transmission electron microscopy | in situ observation

Crystallization can be divided into two processes: nucleation and crystal growth. The crystal growth process has been well examined for a long time, yet the nucleation process is not understood; for example, the nucleation rate of crystals provides a textbook example of order-of-magnitude discrepancies between theoretical predictions and experimental results. Recent proposals have attributed these discrepancies to a nonclassical nucleation pathway, along which a structured crystalline embryo forms within a highly concentrated disordered precursor (1). This mechanism was first proposed for protein crystals (2, 3). Direct observations have demonstrated its applicability to organic (4), inorganic (5, 6), and colloidal (7) crystals. In proteins, clusters of protein molecules have been suggested as precursors; these clusters have mesoscopic sizes from several tens to several hundreds of nanometers and are considered to behave like liquids. It has also been suggested that the precursor is thermodynamically stable with respect to the mother liquid phase but is metastable or unstable with respect to the crystalline phase. The latter nature of the precursor differs from the stable macroscopically dense liquid formed as a result of the liquid–liquid phase separation (8). Such protein-rich mesoscopic clusters have been observed for many proteins, primarily using optical techniques, and have been tentatively identified as precursors for crystal nucleation (9–12). Several important questions concerning this mechanism remain unanswered. First, are the observed mesoscopic clusters actually liquid-like or solid-amorphous? Second, do they play an active role in crystal nucleation? In addition, finally, do the clusters serve as classical heterogeneous nucleation centers or as loci of enhanced nonclassical nucleation? To address these questions, we used time-resolved in situ transmission electron microscopy (TEM) with a liquid cell (Fig. S1) to

directly observe crystal nucleation with the protein lysozyme, arguably the most closely studied enzyme (13).

Results and Discussion

Analysis of the Crystallization Solutions. To accelerate nucleation, the liquid cell was initially charged with an unfiltered crystallization solution (50 mg·mL^{−1} of lysozyme and 5 wt% NaCl in a sodium acetate buffer solution); subsequently, a filtered growth solution (15 mg·mL^{−1} lysozyme and 5 wt% NaCl in a sodium acetate buffer solution) was continuously passed through the liquid cell to maintain a constant concentration. Both solutions were analyzed using dynamic light scattering (DLS) (Fig. S2). The DLS signal from the unfiltered solutions is nearly identical to numerous published datasets collected under similar conditions that reveal the presence of mesoscopic (diameters, >100 nm) clusters, which have been suggested to act as precursors for crystal nucleation (9, 10). These solutions clearly lie outside the liquid–liquid coexistence region of the phase diagram, indicating that stable macroscopic dense liquids should not form under these conditions (8). The DLS characterization of the filtered solutions reveals the presence of only lysozyme monomers (Fig. S2).

In Situ TEM Observations. In situ TEM observations (Fig. 1A) demonstrate particles with a range of sizes similar to those identified using DLS. Most of the particles were immobile because they were attached to the liquid-cell windows. The mean diameter of the particles was estimated directly from the TEM images to be ~170 nm (Fig. S3A). The electron diffraction patterns of the particles exhibit halo rings and no diffraction contrast in the bright-field

Significance

The formation of the nuclei of protein crystals has been suggested to occur within protein-rich mesoscopic clusters. The existence of such clusters has been revealed for many proteins; however, their role in crystallization is still unclear. Our live images in a protein crystallization solution using transmission electron microscopy reveal that protein-rich mesoscopic clusters are solid amorphous particles that work as heterogeneous nucleation sites. The nucleation event for the crystal starts via another noncrystalline particle, which appears only a few seconds before crystal nucleation, that is, there are two types of amorphous particles that have different roles in protein crystallization.

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Fig. 1. The amorphous and crystal phases in supersaturated lysozyme solutions. In situ TEM images of (A) numerous amorphous solid particles (ASPs); (B) an elongated orthorhombic lysozyme crystal; and (C) a tetragonal lysozyme crystal. The corresponding electron diffraction patterns are shown in D–F, respectively. The arrows in B and C indicate the $\langle 001 \rangle$ direction. In C, the $\{110\}$ face of the tetragonal lysozyme crystal faces upward. [Scale bars: 500 nm (A), 1 μm (B), and 200 nm (C).] In E and F, the crystal zone axes are $[10314]$ and $[1-10]$, respectively.

images (Fig. 1D). Furthermore, they did not coalesce even though many of them were in direct contact with each other. Amorphous particles were also confirmed by corresponding diffraction patterns from the cryo-TEM observations (Fig. S3 B and C). Note that the particles could not be observed in the filtered solutions. From these observations, we conclude that the particles are amorphous solid particles (ASPs) of lysozyme. The ASPs are similar in size to the mesoscopic clusters, which have been suggested to be precursors for crystal nucleation, as reported in previous studies (9, 10). However, crystals never formed within the ASPs during our observations.

In multiple experiments, we observed both orthorhombic and tetragonal crystals in addition to the ASPs (Fig. 1). The morphologies of the crystals were similar to those of corresponding macroscopic crystals that form under similar conditions (14, 15). For additional identification, we analyzed the electron diffraction patterns generated by these crystalline phases (Fig. 1 E and F). The long-term evolution of two crystals growing in close proximity (Fig. S4) revealed that the orthorhombic crystals grew while the tetragonal crystals dissolved, indicating that the former are more stable polymorphs under the tested experimental conditions. In addition, we observed orthorhombic lysozyme crystals and ASPs in the same view (Fig. S5). The ASPs dissolved completely as opposed to the stably existing orthorhombic lysozyme crystals, suggesting the lower solubility of the orthorhombic lysozyme crystal than that of the ASPs, that is, orthorhombic crystals are more stable than ASPs under the experimental conditions.

During more than 60 h in 30 separate experiments under optimized experimental conditions, we monitored the nucleation events of an orthorhombic lysozyme crystal attached to a silicon nitride

window (Fig. 2A and Movie S1). First, a spherical particle (indicated by an arrowhead in Fig. 2A) formed. As it grew, the particle attained crystal facets and, finally, transformed into an orthorhombic crystal with a characteristic shape elongated in the $\langle 001 \rangle$ direction. In other experiments, we observed the nucleation of orthorhombic crystals on ASPs (Fig. 2B and Movie S2). A spherical particle (indicated by an arrowhead in Fig. 2B) was observed to nucleate and transform into an orthorhombic crystal. Because the initial particle has a weaker contrast than that of an ASP at the same size, the nucleated particle may have a different internal structure and density, or shape, than that of the ASPs. This observation clearly indicates that the nucleation event did not start within the ASP; instead, the ASP acted as a heterogeneous substrate that enhanced the nucleation event.

To test the role of the ASPs in the nucleation process, we filtered the crystallization solution. TEM and DLS observations (Fig. S2) indicated that the ASPs were removed from the filtered solutions. Then, the nucleation rates were drastically reduced, likely as a result of the lack of heterogeneous substrates (9). Therefore, the participation of the ASPs as heterogeneous substrates is crucial to crystal nucleation in this system.

Evolution of the Growth Rates of Nucleated Particles. For additional insight into the nucleation process, we compared the evolution of

Fig. 2. The nucleation of lysozyme crystals monitored by time-resolved in situ TEM. (A) A spherical particle observed at 0.1 s (yellow arrowhead) transforms into an orthorhombic crystal. A second crystal nucleates at 10 s. The size, L_1 , is defined by two dashed lines and a bidirectional arrow at 1 s. (Scale bar: 200 nm.) (B) A spherical particle, indicated with a yellow arrowhead, forms at 0.17 s near an amorphous solid particle (ASP) and transforms into an orthorhombic crystal. The size, L_2 , is defined similarly to L_1 in A. (Scale bar: 200 nm.)

Nucleation Process of Lysozyme Crystals. To demonstrate the appearance of a crystal within a noncrystalline particle, we show cryo-TEM images in Fig. 4. The ununiformed contrast can be observed from the particle shown in Fig. 4A. A Laue spot in the corresponding diffraction pattern (Fig. 4B), the faceted shape, and the corresponding diffraction contrast (Fig. S10) represent the crystalline phase. These cryo-TEM images may show that a crystal appears within a noncrystalline particle. To precisely prove the appearance of a crystal in a noncrystalline particle, 3D images may be useful because a TEM image is just a projection. In summary, time-resolved in situ TEM observations of supersaturated solutions of the protein lysozyme demonstrated crystal nucleation following a heretofore-unsuspected modification of the two-step mechanism. Based on the observations, the pathways of this mechanism are schematically illustrated in Fig. 5. In contrast to earlier proposals (2, 3, 9–12), we demonstrate the action of two mesoscopic amorphous phases: amorphous solid particles that serve as heterogeneous nucleation substrates and short-lived (approximately 0.1 s) noncrystalline particles, which form on the container wall or the ASPs and host crystal nuclei. Even though the detailed features of the noncrystalline particles are still unclear, a plausible feature is that of a dense liquid because molecules inside the particles have the flexibility to form crystalline structures, which may be a feature of the liquid phase. Importantly, lysozyme molecules do not directly assemble into crystal nuclei and the ASPs never transform into crystals. This observation gives us insights into the nucleation of not only protein crystals but also other organic crystals. Scattering and spectroscopic techniques reveal the structure evolutions of organic materials in solutions and the existence of clusters (25). To understand the nucleation, our observations suggest the importance of the direct observation of nucleation events at the nanoscale to reveal the role of amorphous particles on crystal nucleation.

We demonstrate that the crystals emerging from the non-crystalline particles are rough and follow a normal growth mode, with faceting attained at later growth stages. For additional insights, further analyses of observed noncrystalline particles, such as formation kinetics, are required.

Materials. Six-times-recrystallized lysozyme powder (lot no. E40314; Seikagaku Kogyo) was used as a protein sample without further purification. A 50 mM solution of sodium acetate (pH 4.5) was used as the buffer solution. Stock solutions of lysozyme were prepared by dissolving the appropriate amount of lysozyme powder in the buffer solution. The concentration of the lysozyme stock solutions was measured using UV absorption spectrometry (SmartSpec Plus; Bio-Rad Laboratories). NaCl (99.99%; Wako Pure Chemical Industries) was dissolved in the buffer solution to act as a precipitant for the lysozyme. The crystallization solution was prepared by mixing appropriate amounts of the stock solutions. The lysozyme powder sample contained ~1.5% impurities, which consisted primarily of lysozyme dimer and 18-kDa polypeptide (28).

Observations. The supersaturation of the crystallization solution is defined as $\sigma = \ln(C/C_c)$, where C is the bulk concentration of lysozyme and C_c is its solubility at a given temperature. We used two crystallization solutions with different supersaturations. A crystallization solution with a higher supersaturation was used to initially fill the liquid cell before the observation. This solution was prepared by mixing the lysozyme solution and NaCl solution immediately before filling the liquid cell. The resulting solution contained 50 mg·mL⁻¹ lysozyme and 50 mg·mL⁻¹ NaCl and was not filtered. The crystallization solution, with a lower supersaturation, contained 15 mg·mL⁻¹ lysozyme and 50 mg·mL⁻¹ NaCl, and flowed through the liquid cell during the TEM observations. This crystallization solution was filtered through a cellulose acetate filter with 0.20-μm pores. During the observations, the solution was passed continuously through the liquid cell at a rate of 1–3 μL·min⁻¹ by means of a syringe pump. All of our experiments were performed at 24 °C; as a result, the degree of supersaturation, σ , in the liquid cell was constant. We used values of 3.19 and

3.30 mg·mL⁻¹ as the solubilities at 24 °C for the orthorhombic (29) and tetragonal phases (30), respectively, to maintain consistency with our observations of the stability of these phases (Fig. S4). The supersaturations of the crystallization solution with the lower supersaturation were $\sigma = 1.55$ and $\sigma = 1.51$ for the orthorhombic and tetragonal forms, respectively.

Electron Microscopy. We used two Hitachi transmission electron microscopes: an H-8100 and an HF-3300. The H-8100 with a LaB₆ filament was operated at an acceleration voltage of 200 kV. Images and movies were recorded using an XR-611 TEM camera (Advanced Microscopy Techniques), which has a recording interval of 157 ms. The HF-3300 with a field-emission gun was operated at an acceleration voltage of 300 kV. Movies were recorded using an ORIUS SC1000 CCD TEM camera (Gatan), which had a recording interval of 100 ms. The electron flux was measured using a combination of a probe-current detector and a Model 6485 Picoammeter (Keithley Instruments) installed in the H-8100.

The TEM Holder and the Assembly of the Liquid Cell. We used a TEM holder (Protochips) in combination with a liquid cell to image the liquids. The liquid cell consisted of a pair of semiconductor-based plates with an electron-transparent window consisting of an amorphous silicon nitride membrane and two Viton O-rings. These components permitted the preparation of the sample solution under high-vacuum conditions in the TEM (Fig. S1) and allowed the solution to be passed through the liquid cell by means of a syringe pump during the observations. The plates were separated by 150-nm-thick or 500-nm-thick spacers to form the flow path for the sample solution. The thickness of the observed solution layer was larger than the thickness of the spacer due to the expansion resulting from the vacuum present in the electron microscope (31). Before assembling the liquid cell, the plates were subjected to a hydrophilization treatment using plasma-ion bombardment.

Rates of Growth of the Lysozyme Crystals Examined Using Optical Microscopy. We observed orthorhombic lysozyme crystals using phase contrast microscopy (PCM), and we measured their growth rates for comparison with those observed using TEM. For these measurements, we used a growth cell (32) in which a seed crystal and the growth solution were sealed. Seed crystals for the PCM observations were produced using a crystallization solution (100–120 mg·mL⁻¹ lysozyme plus 25 mg·mL⁻¹ NaCl in the buffer solution). The solution was sealed in a glass tube (~400 mL) and kept in an incubator at 40 °C to produce orthorhombic crystals. After 2–3 d, several crystals measuring 100–200 μm were formed, and these were used as seed crystals. A seed crystal was placed in the cell and aligned with its *c* axis parallel to the focal plane. The growth solution, which contained

15 mg·mL⁻¹ lysozyme and 50 mg·mL⁻¹ NaCl, was identical to that used in the experiments in the TEM. Before the growth solution was sealed in the cell, it was filtered using a syringe filter with a pore size of 0.20 μm. For the growth rate measurements, the temperature of the cell was maintained in the range of 24–28 °C using Peltier elements on the cell stage. The range of supersaturation σ of the solutions was 1.38–1.55 for the orthorhombic crystals. The temperature at which we measured the step velocity for the orthorhombic crystal (Fig. S7) was 24 °C; the corresponding supersaturation σ was 1.55.

DLS Characterization of the Solutions. The correlation functions of light scattered by the tested solutions were recorded using an ELS-Z1TK instrument (Otsuka Electronics) equipped with a semiconductor laser (wavelength, 660 nm). We used a quartz cuvette. The respective correlation functions of these solutions are shown in Fig. S2.

Cryo-TEM Observations. We used a field emission TEM, JEM-2200FS, operated at an acceleration voltage of 200 kV. Images were corrected using a MSC model 794 (Gatan). The sample solution was put on a micro grid, moved to a cryotreatment, and was placed on the cryo-TEM holder. After the treatment, the grid was maintained continuously under -170 °C to prevent crystallization of the water.

Determination of the Size of the ASPs. From the TEM images, we measured the mean diameter of the ASPs in the experimental solution. An example image is shown in Fig. S3. The ASPs were counted, and the surface area occupied by the ASPs in the plane was measured. By assuming that each particle was spherical, we calculated the mean diameter of the ASPs to be 170 nm from the number and total area of the particles.

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