Pattern formation and coarsening during metastable phase separation in lysozyme solutions

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We observed interesting structures during phase transformations of lysozyme solutions. The process begins with the separation of a protein-rich liquid phase in the form of droplets. The droplets fall to the bottom of the chamber in a few minutes, and on the scale of an hour they begin to merge, forming an interconnected spongelike structure. In the final transformation process, the sponge turns into crystals. The existence of the sponge phase depends upon the relative time scales for droplet coalescence and crystal nucleation, something we were able to vary by changing the salt concentration in our solution. We expect our observations to have significance for producing protein crystals for x-ray structure analysis of proteins.

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I. INTRODUCTION

Protein crystals of high quality are indispensable for the determination of three-dimensional structure of protein molecules by x-ray structure analysis. Producing protein crystals, however, is generally difficult partly because the protein solutions exhibit various kinds of phase transition phenomena. These include the crystallization itself and liquid-liquid phase separation [1–3]. These various phase transition phenomena can influence each other, making protein crystallization difficult to predict. For example, it has been experimentally suggested [4–10] and theoretically predicted [11–13] that the liquid-liquid phase separation affects the crystallization process, especially at the nucleation stage. Clear understanding of the relation between the various phase transitions, and of conditions in which crystal nucleation occurs, is necessary for protein crystallization in a well-controlled manner.

Recently, various types of phase separations have been found in polymers and alloys [14–19], some of which cannot be explained by the conventional theory of phase separation [20]. For example, in polymer solutions or polymer mixtures the viscoelasticity of the separated phases was found to affect severely the coarsening process [14–17]. Also, in a solid-solid phase separation of a binary alloy, elastic properties of the two phases can couple with the phase-transformation process [18,19]. These reported phenomena appear only when the dynamic or static properties such as viscoelasticity or elasticity are quite different between the two separated phases.

In the liquid-liquid phase separation of protein solutions the viscoelastic properties are also quite different between a protein-rich phase and a solvent-rich one. Therefore, the process of the phase transformation in a protein solution is expected to be understood by the same concepts used for polymer system described above. There are, however, few studies on the phase-transformation process of protein solutions.

In this paper, we focus on the process of the liquid-liquid phase transformation of lysozyme solutions, which either precedes the crystallization or takes place with it simultaneously. We attempt to explain the whole process of the structure formation using the concept of the difference in viscoelastic properties between the two phases. We also demonstrate below that the competition between the liquid-liquid phase transformation and crystallization can make various transient patterns in the solutions. We expect that the protein crystal growth, an important problem in structural biology, can benefit from this study, which connects the process of the various phase transformations in protein solutions with the existing understanding of the physics of pattern formation in polymers [14–16] and alloys [18,19].

II. MATERIALS AND METHODS

Hen egg lysozyme of molecular weight $M_W = 14\,307$, purified six times by crystallization, was purchased from Seikagaku. High-purity deionized water of about 18 $\mathrm{M}\Omega\,\mathrm{cm}$ from a NANOpure system (Barnstead) was used for all solutions. The pH values were adjusted by 50 $\mathrm{m}M$ Na-acetate buffer at 4.60 ± 0.1.

Liquid-liquid phase separation was induced in lysozyme solutions containing NaCl by lowering the temperature. We prepared aqueous lysozyme and NaCl solutions separately, and mixed them to an appropriate volume ratio. Before mixing, the lysozyme solution was passed through a 0.025 μm filter and the NaCl solution through a 0.2 μm filter (Millipore). The final concentration of lysozyme was fixed at 10.0% (w/v), while the NaCl concentrations varied from $0.8M$–$1.0M$.

The mixing was done at a temperature that is high enough not to cause the liquid-liquid phase separation but is sufficiently below the denaturation temperature. It was followed by injecting the solution into an observation glass cell of 22×22 mm^2 size with a thickness of about 0.2 mm and then lowering the temperature. The temperature was controlled

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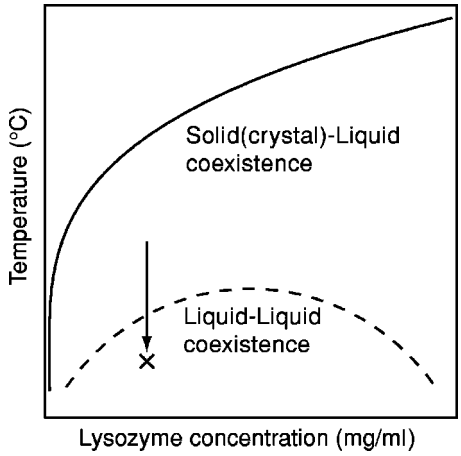

FIG. 1. A schematic phase diagram of lysozyme solutions including a stable solid-liquid coexistence curve (crystal solubility) and a metastable liquid-liquid coexistence curve. Lysozyme solutions in the experiments were quenched as shown by the arrow, and incubated at the point shown by the symbol "×."

within $\pm 0.5\,^{\circ}\mathrm{C}$ by water circulation. The quench (temperature lowering) rate was about $2\,^{\circ}\mathrm{C}$/min. An elapsed time t was measured from the time of mixing.

Figure 1 shows a schematic phase diagram [5] of lysozyme solution, which includes a stable solid-liquid coexistence curve (solid line) and a metastable liquid-liquid coexistence curve (broken line). Observation was done at a point below the liquid-liquid coexistence curve shown by "×" in Fig. 1. The distance from the liquid-liquid coexistence curve to the observation point is "the depth of quench." Experimentally, it can be estimated from the difference in the two temperatures, a cloud point and an observation point.

The separation of protein-rich domains from a uniform solution and their transformation process were observed by an optical microscope (Axiovert S100, Carl Zeiss) and recorded by a digital charge-coupled device imaging system (SenSys, Photometrics). The averaged size of the protein-rich domain was estimated as follows. The number of the boundaries n between the protein-rich and solvent-rich domains were counted on a line drawn from one side to the other of a digital image. The averaged size of domain l was approximately estimated to be $l=2L/n$, where L is the size of the image.

III. RESULTS

Figure 2 shows an example of the droplets of protein-rich phase separated out from the uniform solution containing $1.0M$ NaCl. The cloud point was $25.2\pm 0.3\,^{\circ}\mathrm{C}$ and the depth of quench was $2.1\pm 0.5\,^{\circ}\mathrm{C}$. The separation started within a minute after lowering the temperature. In about two minutes, the separation seemed to be finished with no new droplets forming. The droplets sank to the bottom of the glass cell over the course of about 10 min (piling) and although they stuck together, further fusion (coarsening) did not occur for another 20–30 min.

Figure 3 shows the coarsening process that occurred after the initial droplet formation. The piledup droplets started to

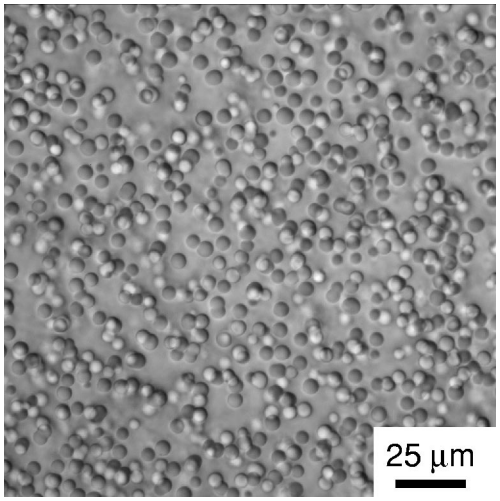

FIG. 2. Droplets separated from a solution. These droplets floated in the solution for about 10 min, and then piled up on the bottom of the glass cell. The lysozyme and NaCl concentrations were 100 mg/ml and $1.0M$, respectively. The cloud point was $25.2\,^{\circ}\mathrm{C}$. The process was observed at $23.1\,^{\circ}\mathrm{C}$.

connect and merge with each other about 40 min after mixing. These connected droplets formed an interconnected spongelike structure (Fig. 3), that was maintained for more than 6 h. The coarsening proceeded as the spongelike domains became thicker [Fig. 3(a) and 3(b)]. Gradually the boundary between the protein-rich and solvent-rich phases became vague due to the collapse of the structure [Fig. 3(c)].

Crystals (spherulites) of lysozyme appeared at the same time as the structural collapse, as shown in Fig. 3(c). Their growth in the same area as shown in Fig. 3 is shown in Fig. 4. As the crystals grew, the boundary of the spongelike structure became even more unclear [Figs. 4(a) and 4(b)] with the spherulites completely taking over the spongelike protein-rich domain [Fig. 4(c)]. Note that the spherulites polarized light while the protein-rich phase or spongelike structure did not. This indicated the ordered structure in the spherulites as opposed to the disordered structure in the protein-rich phase.

Figure 5 shows the change in the averaged domain size l. For about 2 h after the formation of the droplets, during the transformation from the droplets to the spongelike structure, the domain size did not change. After about 3 h after mixing, the domain size started to increase with time. The solid line represents the relation of $l\sim t$.

Figure 6 shows the crystal growth coupled to the vanishing of the droplets in a solution where the concentration of NaCl was slightly lower $(0.88M)$ than the previous case $(1.0M)$. The cloud point was $24.0\pm 0.3\,^{\circ}\mathrm{C}$ at this NaCl concentration and the depth of quench was $3.2\pm 0.5\,^{\circ}\mathrm{C}$. In addition to the spherulites, single crystals also grew in this solution. As shown in Figs. 6(b) and 6(c), the droplets were absorbed by crystals or dissolved as the crystals grew. The droplets did not merge with each other and, therefore, only a vanishing process was observed in this solution. No interconnected spongelike structure was formed.

IV. DISCUSSION

There are two striking features in this phase-transformation process: (1) droplets and spongelike structure

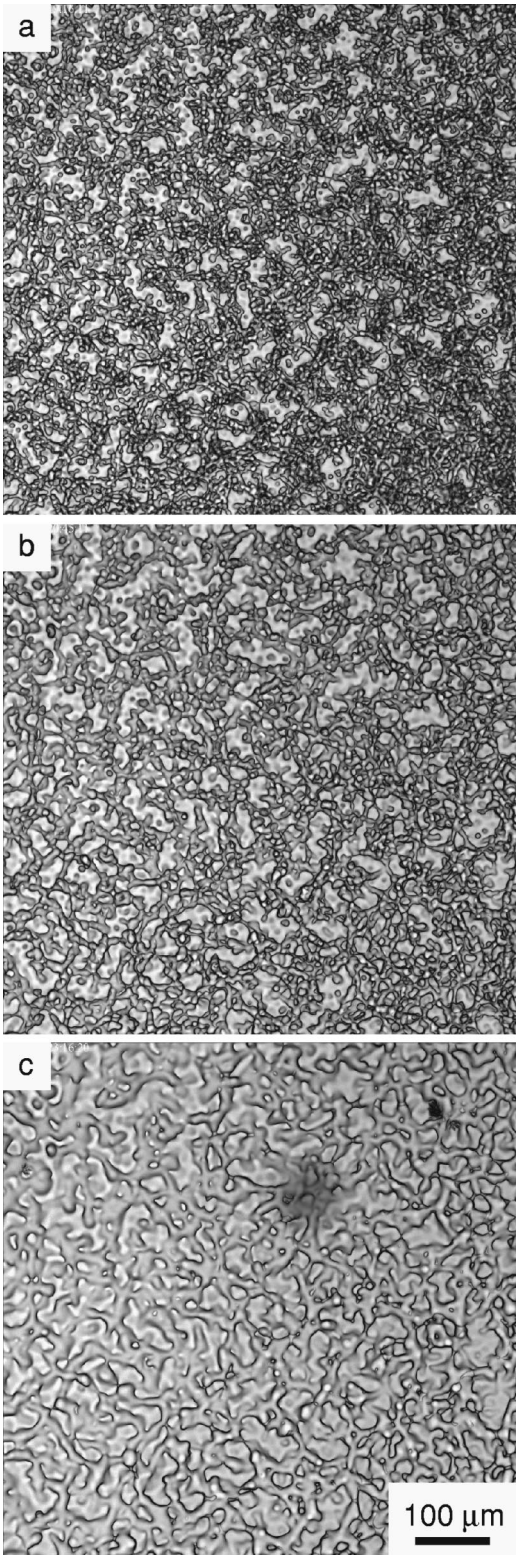

FIG. 3. A spongelike structure and its coarsening process. The solution was the same as the one shown in Fig. 2. The images were taken at (a) 1.7 h, (b) 2.8 h, and (c) 4.8 h after the mixing.

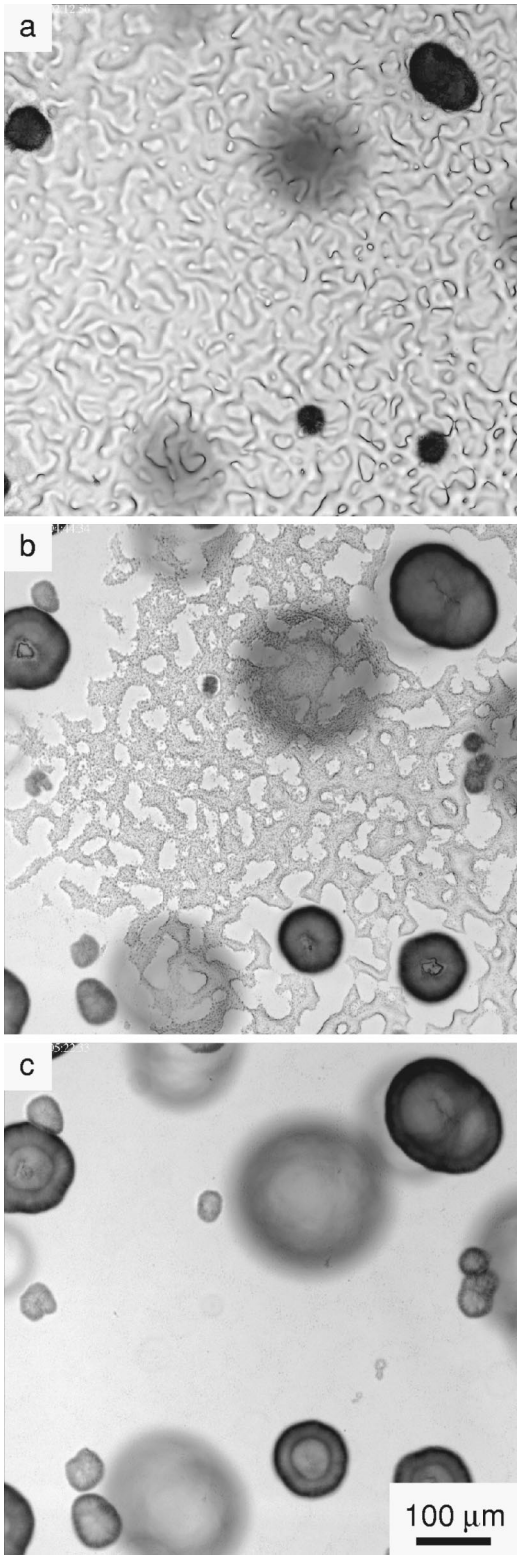

FIG. 4. A vanishing process of the protein-rich domain and the growth of spherulites (dark spherical objects). The solution was the same as the one shown in Figs. 2 and 3. The images were taken at (a) 7.2 h, (b) 9.2 h, and (c) 9.7 h after the mixing.

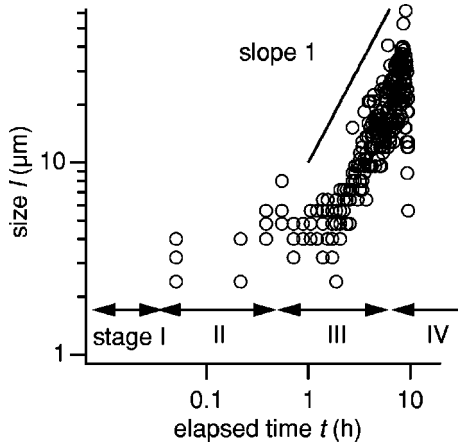

FIG. 5. A double logarithmic plot of a change in the estimated protein-rich domain size l with the elapsed time t. The measurement was done using the same solution as in Figs. 2–4. The solid line represents the relation of $l \sim t^a$ with $a = 1.0$. The whole process of the phase transformation was divided into four stages: Protein-rich droplets separated from the solution (stage I). The protein-rich droplets started piling up on the bottom of the glass cell whereas the coarsening did not proceed (stage II). The coarsening began and the spongelike structure formed (stage III). Crystals started growing and the protein-rich domain vanished gradually (stage IV).

appear sequentially in the same solution, and (2) the liquid-liquid coexistence is not stable but transient since a more stable crystallization event follows. The former indicates that the surface tension does not play a crucial role at least during the transformation of the structure from droplets to a spongelike structure, since a spherical shape like the droplets will correspond to the surface tension minimum. The latter indicates that the liquid-liquid phase transformation and crystallization compete with each other. To see these features in detail, we divided the whole process of the phase transformation into four stages (illustrated in Fig. 5 as stages I–IV). Droplets of the protein-rich phase separate out from the uniform solution in the first stage (Fig. 2). The droplets pile up on the bottom of the glass cell with their spherical shape maintained in the second stage. The droplets connect and form a spongelike structure in the third stage (Fig. 3). The spongelike structure was taken over by the crystal growth in the fourth stage (Fig. 4). We discuss below the mechanism of the phenomena in each stage in detail.

A. Structure change from droplets to spongelike

We consider the viscoelasticity difference between the protein-rich and solvent-rich phases to explain the phenomena on the first, second, and third stages.

1. Pinning of the coalescence of the droplets

In the first stage (Fig. 2), the droplets formed do not fuse even when they collide with each other, and they may move apart again. Tanaka reported for the first time that a coarsening process was virtually prevented in a polymer solution when a separated polymer-rich phase was so dense that its deformation time was much larger than that of the character-

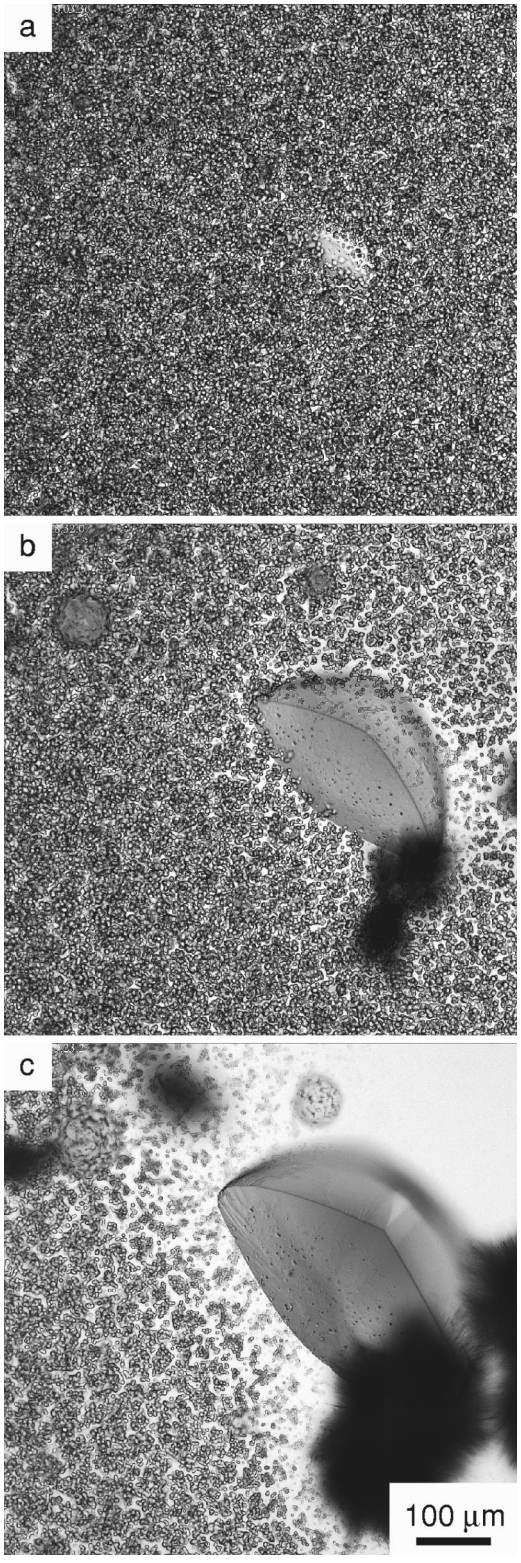

FIG. 6. The competition between the liquid-liquid phase transformation and the crystallization. The lysozyme and NaCl concentrations were 100 mg/ml and 0.88M, respectively. The cloud point was 24.0 °C, and the observation was done at 20.8 °C. In this solution, spongelike structure did not form. Note that the protein-rich droplets were incorporated on the surface of the crystal.

istic collision or contact time [21]. We believe that the same idea can be applied to protein solutions. In our system, droplets of the protein-rich phase are expected to be quite viscous in comparison with the surrounding solvent-rich phase. Therefore, the relaxation against deformation is much faster in the surrounding solvent-rich phase than in the droplets of the protein-rich domain. Then the droplets move around easily, which prevents maintaining the connection between one another.

In the second stage (the stage II of Fig. 5), droplets pile up on the bottom of the glass cell with their spherical shape maintained. Even after this, they do not connect with one another for several tens of minutes. In this stage it appears that the coarsening process is frozen (the stage II of Fig. 5). A similar situation has been observed in the solid-solid phase separation process and is called the pinning effect [18,19]. The pinning is considered to occur when the elastic properties of the two phases were different (elastic misfit) and when a more elastic phase was surrounded by a less elastic phase. In our case we suggest that the coarsening process is prevented because the viscous protein-rich domain is surrounded by the less viscous solvent-rich phase. In other words, during several tens of minutes in the second stage, the droplets behave as elastic bodies, whereas the solvent-rich phase behaves as a fluid.

To explain the pinning behavior on the molecular level, Glotzer *et al.* [22] simulated a coarsening process of the binary fluids where each monomer had two types of nearest-neighbor interactions, strong and weak ones. Considering one strong bond and Ω weak bonds, they showed that in the limit of $\Omega \rightarrow \infty$, the coarsening process was normal. When Ω was small, however, at first, the coarsening proceeded normally but then pinned for a certain time. Based on these results, we suggest that some strong bonds between the molecules are made in our protein-rich droplets concentrated by the phase separation.

2. Formation of the spongelike structure

During the third stage, the coarsening starts (Fig. 3 and the stage III of Fig. 5) and the spongelike structure gradually forms. This means that the pinning is not the final stage in our system, because even the protein-rich domain is a fluid over a long time scale. In other words, the transfer from the second stage to the third is marked by the appearance of viscoelasticity. That is, the solidlike or fluidlike behaviors appear dependent on the time scale of the deformation.

The spongelike structure is the result of random stacking of the droplets. In the coarsening, the thicker part of the protein-rich domain becomes thicker and the thinner part becomes thinner (Fig. 3). This behavior is typical of the percolated phase separation when the volume fraction of the separated phase exceeds the percolation limit [20,23]. In our case, the sedimentation of droplets increases the local volume fraction to the percolation limit although the protein-rich volume fraction in an entire cell is much lower than the percolation limit.

The time course of domain growth in the spongelike structure can also be explained by the conventional theory [20,23]. The basic idea of the theory is that the variation of the radius of the domain tube leads to a pressure gradient along the tube axis, which drives the fluid from the necks to the bulges [20]. Siggia estimated the growth of domain size l as $l \sim (\sigma/\eta)t$, where σ and η are the surface tension and shear viscosity, respectively. In Fig. 5, the size l of the protein-rich phase grew as $l \sim t^a$, where $a = 1.0 \pm 0.1$. This exponent value a corresponds well to the theoretical estimation [20,23]. Therefore, it follows that the coarsening in the third stage proceeds conventionally whereas in the second stage (discussed above) and the fourth stage (discussed in the following section), the process of the phase transformation is not conventional.

In summary, we regard the process of spongelike-structure formation as follows. First, the droplets separate and pile up but do not coarsen because of their high viscosity compared with the surrounding solvent-rich phase. The droplets then begin to connect with each other by the relaxation of their viscoelasticity. Due to the local volume fraction of the protein-rich domain increasing with the sedimentation with time, the structure of the protein-rich domain becomes spongelike. The domain growth is driven by the surface tension only after the formation of the spongelike structure, which leads to the growth law of $l \sim t^a$, where $a \sim 1$. In short, the pinning of the phase transformation until the local volume fraction exceeds to the percolation limit causes the transformation from droplets to the spongelike structure.

B. Competition between phase transformation and crystallization

As shown in Figs. 4 and 6, the protein-rich phase is not stable due to the crystal growth. The instability is also understood using the schematic phase diagram of Fig. 1 in which the liquid-liquid coexistence curve locates under the solid-liquid coexistence curve (the crystal solubility). Therefore, once the crystals appear in the solution, the crystallization competes with the liquid-liquid phase transformation. The spongelike structure as shown in Fig. 3 results from the fact that this competition did not occur for several hours. In fact, as shown in Fig. 6, no spongelike structure forms if the crystallization begins before the coarsening proceeds. Therefore, the structure formed depends on the relation between the two time scales of the liquid-liquid phase transformation and the crystallization.

In lysozyme solutions, there is a tendency where the lower the temperature, the earlier the crystals nucleate and grow. In our case, single crystals were grown at 20.8 °C (Fig. 6), compared to the spherulites that were grown at 23.1 °C (Fig. 4). On the other hand, the protein concentration in the protein-rich phase is larger as the depth of quench increases. In our case, the depth of quench was about 3 °C in the case shown in Fig. 6, whereas it was about 2 °C in the case shown in Fig. 4. As a result, the concentration of the protein-rich phase in Fig. 6 was higher than that in Fig. 4, and hence the coarsening process in Fig. 6 became slower than the case shown in Fig. 4 because of the higher viscosity. Therefore, the protein-rich phase in Fig. 6 did not have enough time to form the spongelike structure as that shown in Fig. 4 before crystals took over the protein-rich phase. Thus, a variety of

patterns could be formed depending on the time scale of the phase transformation and the crystallization.

It is worth noting that the protein-rich droplets were incorporated into the crystal lattice directly on the surface of the crystal as shown in Fig. 6. This means that the dense liquid phase can in fact change into crystal. This fact supports a recent idea of protein crystal nucleation where the formation of the dense liquid domain precedes the nucleation [11].

V. CONCLUSIONS

We observed a metastable liquid-liquid phase-transformation process in lysozyme solutions. At first, protein-rich droplets appeared by lowering temperature, followed by the formation of a spongelike structure. We consider that the origin of the above structure formation is the viscoelastic property of the protein-rich phase, that is, the viscoelastic relaxation from solidlike behavior that causes the pinning of the phase transformation, until the local volume fraction exceeds to the percolation limit, to fluidlike behavior, that allows the droplets to be interconnected and form the spongelike structure. We also observed the competition between the phase transformation and the crystallization, with the patterns formed in the solutions depending on the time scales of these processes.

Even from a solution where a liquid-liquid phase separation took place, crystals could grow. Also, a subtle control of NaCl concentration and temperature could cause the difference between the single-crystal and the spherulitic-crystal growth. The liquid-liquid phase separation is thought to be a common phenomenon among protein solutions as is the crystallization. We believe that understanding the phenomena occurring during the liquid-liquid phase transformation may be crucial to understand the phase behavior of protein solutions, including protein crystallization.

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