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Crystals from Light: Photochemically Induced Nucleation of Hen Egg-White Lysozyme

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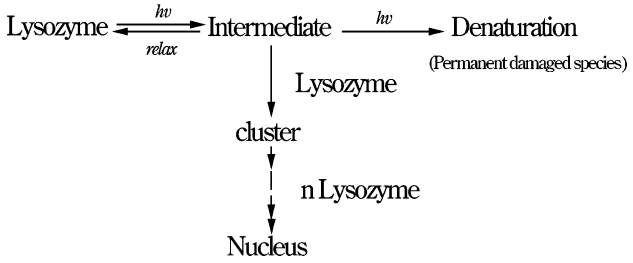
ABSTRACT: In a preliminary study on the light-induced nucleation of lysozyme (Okutsu T.; Furuta, K.; Terao T.; Hiratsuka H.; Yamano A.; Ferté N.; Veesler S. *Cryst. Growth Des.* **2005**, *5*, 1393−1398.), we proposed a nucleation mechanism. In this paper, we demonstrate that the irradiation of a metastable supersaturated lysozyme solution by a light produced by a continuous 150 W Xe lamp induces nucleation and that the mechanism is photochemically induced by a one-photon process. The irradiation of lysozyme molecules produces residual tryptophan radicals, which enhance the attractive interactions between molecules in solution, as shown by dynamic light scattering measurements, and increase the nucleation rate of lysozyme. When the intermediate is excited by visible light, the intermediate is converted into a permanently damaged species, and light-induced nucleation is inhibited, as shown by scavenger experiments (irradiation with two colors of excitation). These results suggest a new method for controlling nucleation and growth from metastable solutions that could be use in structural genomics and the pharmaceutical industry, for instance.

Introduction

Controlling crystal formation, namely, nucleation, is essential for many areas such as production of active pharmaceutical ingredients, protein crystallography, and biomineralization. High-throughput screening,[1,2] that is, incremental variation of all the crystallization parameters to identify crystallization conditions, is time- and materials-consuming. Moreover, the process, from the first crystal appearing in a crystallization trial to the resolution of the crystal structure, may be difficult and lengthy. Growing crystals in the metastable zone, in which nucleation does not occur, is the best method to obtain high quality single crystals without lattice defects. Thus, one strategy is to form a nucleus at a higher supersaturation value and to grow the nucleus at a lower supersaturation value. Controlling supersaturation to set up adequate conditions for nucleation and growth is a challenging task. At present, temperature control techniques are sometimes used to control nucleation and growth,[3−6] and seeding in metastable solutions is often effective,[3] though difficult. An alternative solution is to induce crystallization from metastable solutions using an external energy field, for instance, magnetic,[7,8] electric,[9−11] or electromagnetic.[12−18] Turning to the electromagnetic field, two types of light-induced nucleation mechanisms, photochemical and photophysical, are known. Photochemical-induced nucleation in solution was first reported by Tyndall[12] and in vapor phase has been reported by Tam et al. as "laser snow".[13] Recently, we demonstrated laser-induced morphology control of organic crystals by a photochemical method.[14,18] Photophysical light-induced nucleation and control of polymorphism of supersaturated aqueous glycine solutions, caused by the Kerr effect, have been also reported.[15−17] Recently, laser-induced growth of protein crystals with an intense femtosecond laser (gigawatt) has been reported.[19] The mechanism of the nucleation is thought to be photophysical.

Chart 1. Reaction Mechanism of Photochemically Induced Nucleation[20]



Here we report light-induced nucleation of lysozyme by a continuous xenon lamp. We demonstrate that nucleation is photochemically enhanced and demonstrate the following mechanism: intermediates produced by one-photon absorption produce lysozyme radicals, which enhance nucleation (Chart 1). Hen egg-white lysozyme was selected because lysozyme is widely used as a model system for crystal growth studies and because it is easily available.

Results and Discussion

Light-Induced Nucleation of Lysozyme. We first observed that light irradiation of 30 mg mL^{-1} lysozyme solutions produces white turbidity in solution containing NaCl at 0.5 and 1.0 M. The lysozyme solutions were irradiated for 120 s by light from a Xe lamp; half of the solution was masked by a black paper. Figure 1a−c shows photographs of the lysozyme solutions in this setup. In the lysozyme solution without NaCl, no apparent change is observed (Figure 1a), whereas in the solutions containing NaCl (Figure 1b,c), white turbidity appears in the irradiated part. When the NaCl concentration is increased, the white turbidity is more marked. Because NaCl is known to act as a salting-out agent for lysozyme crystallization, the white turbidity is thought to be aggregates or nuclei of lysozyme. This experimental result suggests that Xe lamp irradiation induces aggregation or nucleation of lysozyme. In addition, when the solutions were observed under optical microscopy, no change (crystal growth and/or Oswald ripening) was noticed in the solution.

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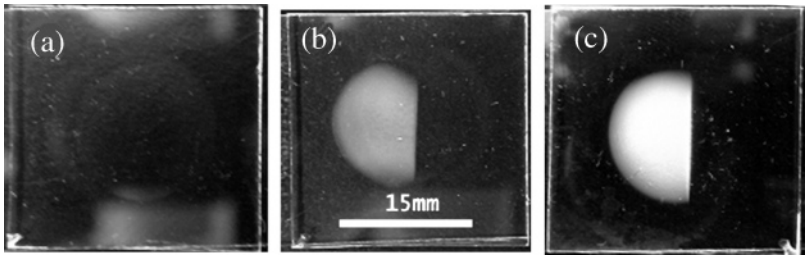

Figure 1. Photographs of irradiated 30 mg mL^{-1} lysozyme solution by Xe lamp for 120 s on slide glasses. Half of the solution was covered with black paper. Concentrations of NaCl were 0 M (a), 0.5 M (b) and 1.0 M (c).

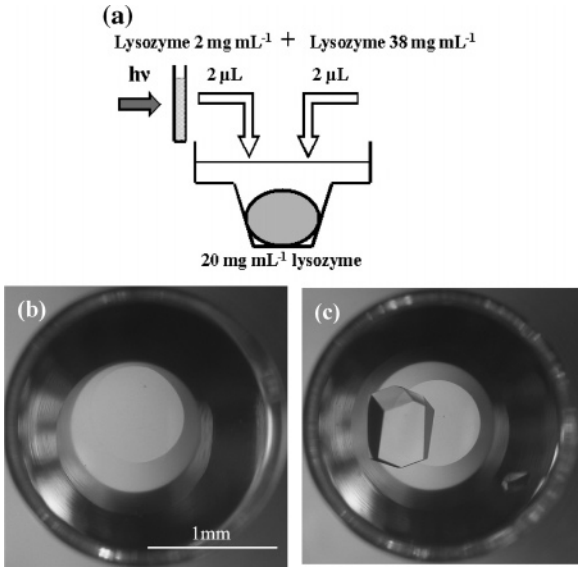

Figure 2. Principle of "liquid-seeding" technique: 2 μL of the irradiated solution is mixed with 2 μL of a concentrated lysozyme solution (38 mg mL^{-1}). The final solution at 20 mg mL^{-1} is stored at 22 °C for 24 h (a). Photographs of the droplets covered with inert paraffin oil using the "liquid-seeding" technique (b) without and (c) with irradiation, 24 h later.

In a previous study,[20] we showed that nucleation is enhanced using hen egg-white lysozyme hanging drop crystallization experiments under light produced by a 300 W continuous Xe lamp. Irradiating supersaturated protein solutions in NaCl at pH 4.5, $\beta = 9$, ($\beta = C/C_e$ is the supersaturation with C and C_e the concentration and solubility of lysozyme) from 10 to 60 s increased the number of lysozyme crystals in the droplet. The most effective irradiation time is from 10 to 30 s, and in this range, enzyme activity is not affected. However, some problems remain in this experimental procedure. First, we must consider the effect of evaporation at the surface of the droplet during the irradiation, which increases the supersaturation of the solution. Moreover, the presence of a solution−vapor interface is reported to induce undesired heterogeneous nucleation,[21] which must be avoided. Second, the optical density of a concentrated lysozyme solution is very high, ca. 60 at 280 nm. This means that the incident light is totally absorbed and scattered within a few tens of micrometers after the solution−vapor interface. Thus, the solution is not uniformly irradiated.

We therefore carried out nucleation experiments using a "liquid-seeding" technique (Figure 2a) The principle of the experiment is the following: 0.5 mL of a 2 mg mL^{-1} lysozyme solution containing 1.4 M NaCl in a 50 mM NaAc buffer solution at pH 4.3 placed in a 0.2 cm × 1 cm × 4 cm optical cell hermetically closed to prevent evaporation was irradiated for 60 s by light from a Xe lamp. Two microliters of the irradiated solution, namely, the liquid seed, was mixed with 2 μL of a concentrated lysozyme solution, 38 mg mL^{-1}, in a 50 mM NaAc buffer at pH 4.3. Thus, the final solution was 20 mg mL^{-1} lysozyme and 0.7 M NaCl at pH 4.3 in a buffer solution at a final supersaturation of 7.[22] In addition, a control solution was prepared with a nonirradiated lysozyme solution. Finally, to avoid undesired heterogeneous nucleation due to evaporation from the vapor−solution interface of the droplets, we used an inert paraffin oil to cover the droplets. The solution was pipetted through the oil. The microbatch plate was kept at 22 °C to avoid nucleation during the growth process. Figure 2b,c shows the photographs of the droplets without and with irradiation, respectively, 24 h later. Sixteen experiments were simultaneously carried out in both cases. In the control solution, no crystal was observed in any of the droplets, suggesting that the solution was metastable and that undesired heterogeneous nucleation at solution−oil interface or solution−wall-of-the-vessel interface was completely suppressed. In the droplets mixed with irradiated solution, tetragonal lysozyme crystals appeared in all the droplets. These results show unambiguously that light induces nucleation of lysozyme. Furthermore, we recently obtained the same results with thaumatin and beef liver catalase.

Dynamic Light Scattering Experiment. Our aim in this contribution is to clarify the mechanism of the light-induced nucleation of lysozyme. For this purpose, we carried out dynamic light scattering (DLS) experiments. DLS experiments give the apparent diffusion coefficient of solute, which is governed by direct thermodynamic and indirect hydrodynamic interactions. If the light irradiation induces nucleation, the potential between solutes is expected to change, becoming attractive. Lysozyme at pH 4.3 is positively charged, +11 charges,[23] and without a crystallization agent such as NaCl, nucleation does not take place due to repulsive interactions between lysozyme molecules in solution. We first prepared lysozyme solution containing 0.2 M NaCl at pH 4.3 to screen protein charges, thereby reducing the repulsive interactions. The results of DLS experiments on these solutions are shown in Figure 3 as closed squares. Diffusion coefficients are plotted against the lysozyme concentration. The slope, k_D, is almost zero, which indicates weakly repulsive interactions between lysozyme monomer molecules in solution. When the sample solution was irradiated for 10 s, k_D changed to a negative value (open squares in Figure 3), indicating attractive interactions between lysozyme monomer molecules in solution. This experimental result shows that light irradiation, which produces intermediates (Chart 1), changes interactions between lysozyme molecules from weakly repulsive to attractive. The experimental fact that interaction potential is attractive between intermediates, namely, neutral tryptophanyl radical, remains to be clarified. Since it has been shown that the intermolecular interactions change from repulsive to attractive when the crystallization

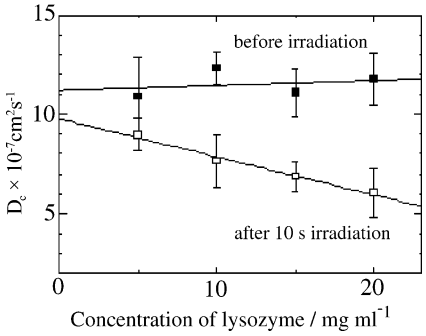

Figure 3. DLS experiments as a function of lysozyme concentration before irradiation (■) and after 10 s of irradiation (□). All the solutions contain 0.2 M NaCl. Error bars are two standard deviations from the calculated mean of the data series

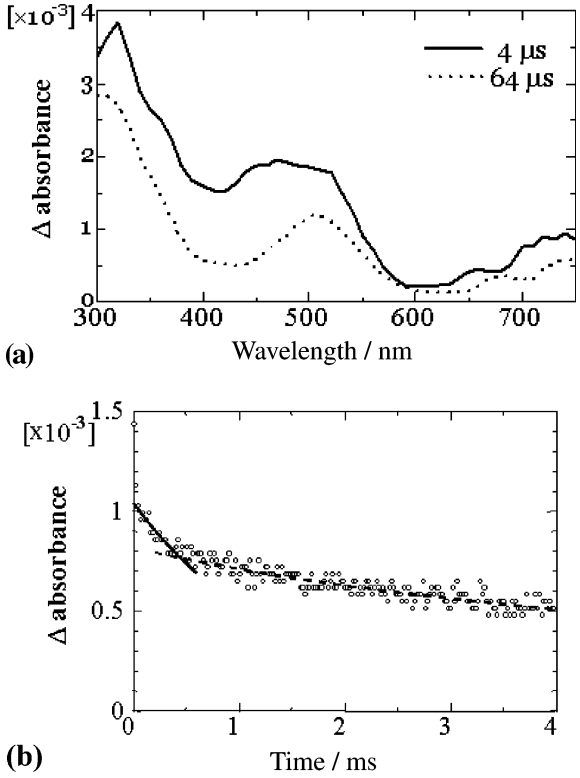

Figure 4. (a) Transient absorption spectra of lysozyme in sodium acetate buffer solution at pH 4.3 (spectra are recorded 4 and 64 μs after the laser flash) and (b) decay time profile of the transient absorption at 520 nm.

conditions are approached,[24,25] we conclude that light irradiation induces nucleation.

Two-Color Excitation Experiments. With a view to identifying the intermediate of Chart 1 and its lifetime, we performed transient absorption experiments. To avoid denaturation of lysozyme by a two-photon absorption mechanism (one for producing a radical and one for denaturing a radical), sample solutions were flowed rapidly (40 mL min^{-1}) through a quartz small cavity cell. Figure 4a shows the transient absorption spectra of lysozyme in 50 mM NaAc buffer at pH 4.3 in O_2 saturated solution. Spectra were recorded 4 and 64 μs after the laser flash. Since the solution was bubbled by oxygen gas, a triplet state was not observed.[26] The spectra are composed of two different species; one is a fast decaying species having absorption in the range 300−600 nm. The fast decaying species is assigned as cystine electron adducts from the literature.[27] The other is a long-lived species having absorption peaks at 320

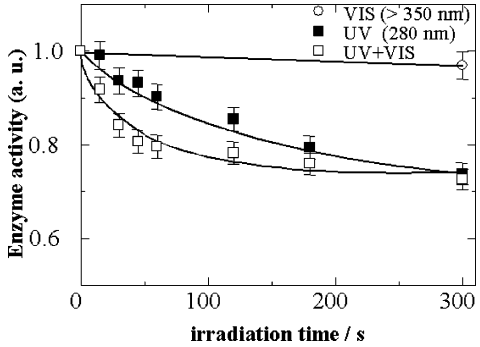

Figure 5. Enzyme activity dependence of lysozyme versus the irradiation time (a.u. = arbitrary unit): irradiated with only visible light (>350 nm), irradiated with only UV (280 ± 10 nm), and irradiated with visible light (>350 nm) and UV (280 ± 10 nm) simultaneously. Continuous lines are only guidelines. Error bars are estimated ±3% for each experimental data.

and 520 nm. The decay time profile at 520 nm is shown in Figure 4b. The decay time profile could be analyzed by a sum of a fast-decaying single-exponential function and a slow-decaying second-order function. The slow component decays through a second-order process indicating that the long-lived intermediate species is a radical. A previous study on lysozyme transient absorption assigned this intermediate species to a tryptophanyl radical.[27] Thus, we consider that the "intermediate" in Chart 1 is a tryptophanyl radical.

To confirm the photochemical mechanism proposed in a previous work[20] and summarized in Chart 1, we performed scavenger experiments to quench the intermediate. Since the intermediate has been assigned to a tryptophan radical (TR)[27,28] and has an absorption band ranging from 300 to 600 nm as shown in Figure 4 from transient absorption experiments, the irradiation of the TR by visible (VIS) light from a 500 W Xe lamp filtered by a cutoff filter (>350 nm) converts TR into a permanently damaged species, making it innocuous for the nucleation process if our assumption is right. In parallel, enzyme activity measurements by ML method as a function of irradiation time with two colors of excitation were carried out by visible light from the 500 W Xe lamp filtered by a cutoff filter (>350 nm) or by UV monochromated (280 ± 10 nm) light from the 300 W Xe lamp or both. Figure 5 shows the variation in enzyme activity against irradiation time for different experimental conditions. Irradiation by visible (>350 nm) light did not change enzyme activity up to 300 s of irradiation (within the experimental error). As obtained previously,[20] for the first 30 s of irradiation by UV (280 ± 10 nm) light, there was no change in enzyme activity, which decreased when irradiation continued longer than 30 s. And last, irradiation by both UV (280 ± 10 nm) and visible (>350 nm) lights decreased greatly enzyme activity after 10 s of irradiation. The facts that the intermediate has an absorption band ranging from 300 to 600 nm, that irradiation by visible light alone does not denature lysozyme, and that coupling UV to visible light increases the efficiency of the denaturation process of lysozyme confirm the two-step deactivation denaturation mechanism proposed in Chart 1.

Finally, to confirm whether the intermediate induces nucleation as suggested in Chart 1, we carried out four nucleation experiments with or without irradiation by UV (280 nm) or visible (>350 nm) lights or a combination of the two as described as "liquid-seeding" technique. Figure 6 shows photographs of droplets under paraffin oil. Figure 6a shows a droplet without irradiation. No crystal appears in the droplet. Figure 6b shows a droplet irradiated by UV (280 nm) light for 120 s.

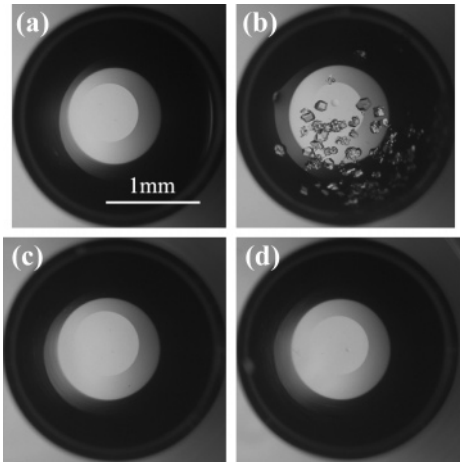

Figure 6. Photographs of the droplets dropped on the microbatch plate covered with inert paraffin oil: (a) without irradiation, (b) with 280 ± 10 nm irradiation for 60 s, (c) with only visible light (>350 nm) for 60 s, and (d) with simultaneous irradiation of visible light (>350 nm) and UV (280 ± 10 nm).

The irradiation time is somewhat longer than the experiments without using monochromated light because the light intensity decreases by passing through the monochromator. As a result, several crystals appear in the droplet, indicating that light-induced nucleation has occurred. Figure 6c shows a droplet irradiated by visible (>350 nm) light. Lysozyme has no absorption in the visible (>350 nm) wavelength range; this result is the same as the result without irradiation. Finally, Figure 6d shows simultaneous irradiation by UV (280 nm) and visible (>350 nm) lights. No crystal was observed in any droplets, confirming the quenching of the intermediate by visible light. These results confirm the photon-induced nucleation mechanism proposed in Chart 1.

Conclusion

Our experimental results clearly show that the irradiation of a metastable supersaturated lysozyme solution by a light produced by a continuous 150 W Xe lamp induces nucleation and that the mechanism is photochemically induced by a one-photon process. The most effective irradiation time is from 10 to 60 s, and in this range, enzyme activity is not affected. The irradiation of lysozyme molecules produces tryptophan radicals, which enhance the attractive interactions between molecules in solution, as shown by DLS, and increase the nucleation rate of lysozyme. When the intermediate is excited by visible light, the intermediate is converted into a permanently damaged species and photochemically induced nucleation is inhibited. From these results, we propose a new method for controlling nucleation and growth from metastable solutions. For instance, a photochemically induced nucleation from metastable solution leads to growth of crystals for structural determination in genomics. Another example, in the case of polymorphism, is that this method could replace seeding by the desired polymorph[29] to enhance the nucleation of the stable polymorph at low supersaturation.

Experimental Section

Preparation of Protein Solutions. Hen egg-white lysozyme was purchased from Seikagaku (6 times recrystallized lot E02Z04) and was used without further purification. Sample solution was centrifuged and filtered through a 50 μm single-use membrane (Millipore) before use. Activity of the lysozyme solution was determined by *Micrococcus lysodeikticus* (*luteus*) cell assay test (ML method). This assay is based on the decrease of absorption at 450 nm when the cells are digested. We refer to the experimental procedure described in the literature.[30] *Micrococcus lysodeikticus* (lot 052K8618) was purchased from Sigma industry.

Light Irradiation. The light source used for the irradiation was a Xe short arc lamp (USHIO UXL-150D, 150 W). The beam from the lamp goes through a quartz lens ($f = 100$ mm, 50 mm diameter), which produces a parallel beam but not focused, and was reflected on the sample by an aluminum-coated mirror. The Xe lamp radiates from 200 nm to the IR region. The power of the UV radiation shorter than 300 nm, which plays an important role for light-induced nucleation, is 30 μW cm^{-2}. Temperature increase of the sample solution was measured with a small thermistor (1 kΩ).

For two-color irradiation experiments, two Xe lamps, a 500 W (USHIO UXL 500D) with a monochromator (JASCO CT-10) and a 300 W (USHIO UXL 300D) with a cutoff filter, were used.

Nucleation and growth by microbatch experiments was carried out by using 72 well microbatch plates (Hampton Research). After the irradiation, all the samples were kept at 20 or 22 °C. Inert paraffin oil (Hampton Research) was used with microbatch plate.

Transient Absorption Measurements. For transient absorption measurements, a Nd^{3+}:YAG laser (Quanta-Ray GCR-130, 30 ns fwhm, 10 mJ pulse^{-1}, 10 Hz) was used as an excitation light source. The samples were flowed through a quartz cell with a flow rate of ca. 40 mL min^{-1}. The transient signals were detected by a photomultiplier tube. The output signals were measured by a digital oscilloscope (Sony Tektronix TDS380P) and transferred to a personal computer. Detailed experimental setup of the transient absorption experiment was described in the literature.[14] Steady-state emission was recorded on a HITACHI F4500 fluorescence spectrometer, and the absorption spectrum was recorded on a HITACHI U3300 spectrophotometer.

Dynamic Light Scattering. Dynamic light scattering (DLS) experiments were performed on a SEM 633 light-scattering apparatus (Semitech, Nice) with an argon ion laser running at a power ranging from 50 to 200 mW and operating at 514.5 nm (Spectra Physics 2017). The measurements were performed at 90° and were processed through a multi-τ correlator (UNICOR) with different sample times between 0.1 and 1.0 μs. Before each measurement, the sample was centrifuged, filtered through a 0.5 μm Millex LCR single-use membrane (Millipore), and introduced in a 12 mm diameter cylindrical glass cuvette; the sample volume was about 300 μL. Solvent density and viscosity were taken equal to the water values, that is 1. Diffusion coefficients were computed by the method of cumulants, and expansion was carried out either the first or the second order. In both cases, the first cumulant was extracted. At each concentration, experiments were performed at different sampling times ranging 0.1 to 1.0 μs. Diffusion coefficients were plotted versus sampling time. The intercept at $\tau = 0$ gave a precise value for the diffusion coefficient. This procedure was previously described by Walrand.[31] The diffusion coefficients D obtained from the cumulant method are apparent diffusion coefficients. They must be extrapolated to zero protein concentration to obtain the free particle diffusion coefficient, D_0. In solutions made of monodispersed particles and in the presence of interactions between particles, the apparent diffusion coefficient is dependent on the concentration C: $D = D_0(1 + k_D C)$. With attractive interactions, the coefficient k_D, the interaction parameter, is expected to be negative, whereas with repulsive interactions, it is positive.

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