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Photochemically induced nucleation in supersaturated and undersaturated thaumatin solutions

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Abstract

We demonstrate photochemically induced nucleation in supersaturated and undersaturated solutions of thaumatin. The irradiation of supersaturated and undersaturated thaumatin solutions in 0.75 M potassium sodium tartrate (PST) at pH 5.6 in *N*-(2-acetamido)iminodiacetic acid (ADA) buffer with $\beta = 2$ or $\beta = 0.5$ increased the number of thaumatin crystals in the droplet. The photochemical reaction induced the formation of unstable small clusters, which led to the growth of nuclei. The mechanism of nucleation enhancement was investigated in steady state and transient absorption experiments. The photochemical intermediate, in which one tryptophan residue is changed to a neutral tryptophanyl radical, appears to be responsible for the nucleation of thaumatin, as well as for the photochemically induced nucleation of lysozyme.
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1. Introduction

Photochemical or photophysical light-induced nucleation has been reported previously [1–4]. Recently, photophysical light-induced nucleation of proteins to generate nuclei from metastable solutions has been successfully achieved with water-soluble [5–9] and membrane-associated protein systems [10].

Previously, we reported photochemically induced nucleation of hen egg-white lysozyme under light illumination at 300 W with a continuous Xe lamp [11]. The irradiation of supersaturated protein solutions in NaCl at pH 4.5, $\beta = 7$–10, for 10–60 s increased the number of lysozyme crystals in the droplet. The most effective irradiation period was 10–30 s; in this range, enzymatic activity was not affected. Enhancement of nucleation by light has been found to be dependent upon the irradiation wavelength associated with the electronic transition of lysozyme. The resulting crystal lattice parameters are identical to those obtained for the original lysozyme crystal.

In our preliminary report, we revealed that photochemically induced nucleation is due to intermediates produced by one photon absorption, which in turn generate lysozyme radicals that enhance nucleation [12]. The intermediate is a protein in which a tryptophan residue is changed into a neutral tryptophanyl radical. Dynamic light scattering measurements have shown that the irradiation of lysozyme molecules produces residual tryptophan radicals, which enhance the attractive interactions between molecules in solution and increase the nucleation rate of lysozyme. Scavenger experiments (irradiation with two colors of excitation) have shown that when the intermediate is excited by visible light, it is converted into a permanently damaged species, and photochemically induced nucleation is inhibited. Thus, it is clear that the intermediate is responsible for the photochemically induced nucleation.

These results suggest a new method for controlling nucleation and growth from metastable solutions that could be use in studies of structural genomics and the pharmaceutical industry.

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The next step in our study is to examine whether photochemically induced nucleation is a general phenomenon for water-soluble proteins. The following proteins were selected for this purpose:

(1) Lysozyme analogue protein, which has a tryptophan (Trp) residue, from which the emission from the protein originates. The nucleation mechanism is expected to be the same as that for lysozyme.
(2) Proteins that lack Trp residues are interesting and very important. The most appropriate candidate is ribonuclease A.
(3) Heme proteins achieve an excited state energy that is lower than the excited energies of amino acids, such as Trp, Phe, and Tyr. The excited state of heme protein is equivalent to the excited state of Fe-porphyrin. If photochemically induced nucleation can be observed in these systems, the mechanism is of great interest. The appropriate candidates are catalase and myoglobin.

Our aim was to demonstrate the nucleation of a lysozyme analogue. Thaumatin was selected as the procedure for its crystallization is well known [13]. The thermodynamic parameters of thaumatin crystallization have been investigated by Kadri et al. [14]. Thaumatin has also been used as a prototype protein in discussions regarding practical crystallization by the light-scattering method [15]. Since thaumatin contains three tryptophan residues [16], photochemically induced nucleation is expected to occur via the same mechanism as that described for lysozyme. The photochemically induced nucleation and photochemical reaction of thaumatin were investigated by steady state and transient spectroscopy.

2. Experimental

Thaumatin was purchased from WAKO Chemicals and used without further purification. Potassium sodium tartrate (PST) was used as the salt, and *N*-(2-acetamido)iminodiacetic acid (ADA) was used as the buffer (both from Hampton Research). Tryptophan, tyrosine, and phenylalanine were G. R. grade (WAKO Chemicals) and were used without further purification. The sample solution was centrifuged and filtered through a 50 μm single-use membrane (Millipore) before use.

The light source used for irradiation was a Xe 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 that is not focused. The Xe lamp emits radiation in the wavelengths from 200 nm to the IR region. The power of the UV radiation with wavelength shorter than 300 nm, which plays an important role in photochemically induced nucleation, was 30 μW cm^{-2}.

Nucleation and growth in micro-batch experiments were carried out in 72-well micro-batch plates (Hampton Research). After irradiation, all the samples were kept at 20 or 5 °C. Inert paraffin oil (Hampton Research) was used with each micro-batch plate.

For the transient absorption measurements, the Nd^{3+} YAG laser (Quanta-Ray GCR-130, 266 nm, 30 ns FWHM, 1 mJ pulse^{-1}, 10 Hz) was used as the excitation light source. The samples were flowed through a quartz cell at a flow rate of ca. 40 mL min^{-1}. The concentration of thaumatin in the solution used for transient absorption measurement was 0.5 mg mL^{-1} (2.3×10^{-5} M). Transient absorption measurements were carried out at room temperature. The transient signals were detected using a photomultiplier tube. The output signals were measured using a digital oscilloscope (Sony Tektronix TDS380P) and transferred to a personal computer. The detailed experimental setup for the transient absorption experiments has been described previously [17]. Steady-state emission was recorded in a Hitachi F4500 fluorescence spectrometer and absorption spectra were recorded in a Hitachi U3300 spectrophotometer.

For hanging drop experiments, 24-well crystallization plates (VDX plate; Hampton Research) were used.

3. Results and discussion

3.1. Solubility of thaumatin

The crystallization of thaumatin for the determination of solubility curves was carried out in sitting drops (50 μL/trial) that were in equilibrium with the precipitating agent solution in the reservoir. Thus, 25 μL of thaumatin (130 mg mL^{-1}) in ADA buffer and 25 μL of PST solution (at concentrations of 0.5, 0.6, 0.7, 1.0, 1.2, and 1.5 M) were mixed on the sitting drop bridge. Two millilitres of 2× PST solution in the same buffer were poured onto the VDX plate. The cavities of the VDX plate were sealed under a cover glass with silicon grease. For each concentration of the salt solution, four simultaneous experiments were carried out. The concentrations of the thaumatin solutions were determined by absorption measurement, with the absorbance at 280 nm corresponding to 2.6 mg^{-1} mL^{-1} cm^{-1} [14].

Fig. 1(a) shows an example of the kinetics of thaumatin concentration changes. The concentration decreased exponentially with time and converged almost to the equilibrium condition after 10 days. The thaumatin concentration after 27 days was used as the solubility value for a specific salt concentration. Fig. 1(b) shows the solubility curves that were determined at three different temperatures. Solubility S is known to be expressed by: $\ln S = \beta + KC$, where β is the intercept with the ordinate and C is the concentration of the electrolyte. The experimental results fit well with this equation [18].

3.2. Photochemically induced nucleation

We carried out photochemically induced nucleation experiments at supersaturated and undersaturated solutions. Nucleation experiments were carried out using the seeding technique. The experimental procedure is shown in Fig. 2. Two solutions were prepared. The solution used for photochemically induced nucleation contained dilute thaumatin (20 mg mL^{-1}) and PST (0.75 M) in 50 mM ADA buffer (supersaturation = 2). The supersaturation value of the solution is shown in Fig. 1(b) with the

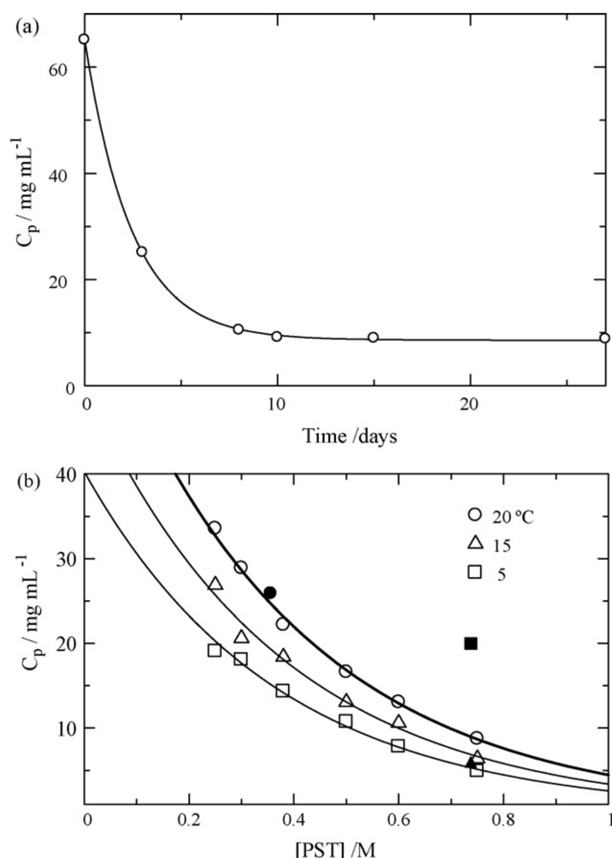

Fig. 1. (a) Time dependence of the concentration of thaumatin at [PST] = 0.75 M. The thaumatin concentration decreased exponentially, and the solubility of thaumatin was determined as the concentration at 27 days. (b) Solubility curve of thaumatin at 20, 15, and 5 °C against PST concentration. The solutions indicated with (■) for supersaturation and with (▲) for undersaturation at 20 °C were irradiated, and these solutions were mixed with concentrated thaumatin solution and stored at the supersaturation level indicated by (●).

■ symbol. The solution used to grow the nuclei was a concentrated thaumatin solution (30 mg mL^{-1}). The former solution was irradiated for 180 s in an optical cell with dimensions of 0.2 cm × 1.0 cm × 4.0 cm. After irradiation, 2 μL of the solution were mixed with an equal amount of concentrated thaumatin solution in a micro-batch plate. The final solution was supersaturated (25 mg mL^{-1} in 25 mM ADA at pH 5.6) and the supersaturation value is indicated in Fig. 1(b) with the ● symbol. Twelve simultaneous experiments were carried out for each condition.

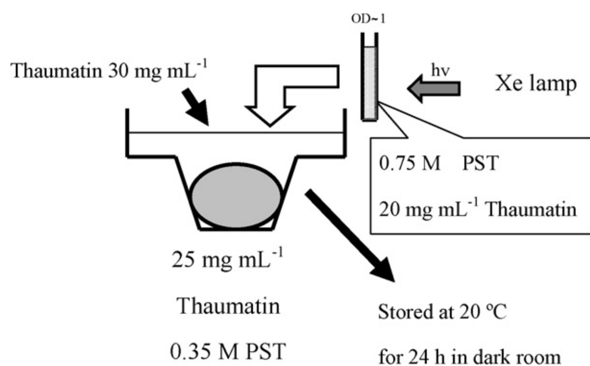

Fig. 2. Experimental procedure for photochemically induced nucleation of thaumatin.

Fig. 3(a) shows photograph of droplets of mixed non-irradiated solutions observed 24 h after the preparation stored at 20 °C in a dark room (control experiment). Bi-pyramidal crystals were observed with apex-to-apex ratios of ∼2, which is the same as that reported for thaumatin crystals [12]. Since the control experiment was designed to examine the effect of photochemically induced nucleation, it was preferable to obtain results without any crystals in the droplet. To obtain this type of metastable condition, we varied both the thaumatin and salt concentrations. Unfortunately, we could not find a metastable condition in which supersaturation occurred without spontaneous crystallization. Spontaneous crystallization is thought to occur due to impurities that cannot be removed by recrystallization or dialysis. Thus, we had no other choice but to treat the number of crystals observed in the control experiment as the baseline value.

Fig. 3(b) shows a photograph of the irradiated solution, with more and smaller crystals appearing in the droplet. The morphology of the crystals is identical to that of the crystal in the non-irradiated solution. The volume of the crystals was about eight-fold smaller than the volume of the crystals obtained from the non-irradiated solution. When the irradiation wavelength was restricted by a cutoff filter to the visible wavelength (>400 nm), the number and size of the crystals were identical to the corresponding values in the control experiment. Thus, photochemically induced nucleation was observed for thaumatin system as well as for lysozyme.

Irradiation of the undersaturated solution was carried out. An undersaturated solution with $\beta = 0.5$ at 20 °C ($C = 5$ mg mL^{-1} where $C_e = 10$ mg mL^{-1} in 0.75 M PST in 0.1 M ADA buffer) was prepared. The concentration of the solution is indicated in Fig. 1(b) with the ▲ symbol. The solution was irradiated for 180 s at 20 °C. The irradiated solution was mixed with a concentrated thaumatin solution (50 mg mL^{-1}) as droplets in micro-batch plates, to grow nuclei for crystals under conditions of supersaturation. Two micro-batch plates were prepared. One plate was stored at 20 °C, while the other plate was stored at a higher supersaturation level at 5 °C for 24 h. The results obtained for the plates stored at 20 °C are shown in Fig. 4(a and b). The two photographs for the non-irradiation and irradiation conditions are almost identical. In both solutions, several crystals appeared. The number and size of the crystals were the same for each condition. No effect of photochemically induced nucleation was noted in this experiment. On the other hand, the effect of irradiation was seen in the solution stored at 5 °C. Fig. 4(c and d) shows the photographs of the non-irradiated and irradiated droplets, respectively. In the non-irradiated droplet, several thaumatin crystals appeared. Fig. 4(d) shows that in the irradiated solution, the number of crystals was higher and the crystals were smaller compared to the crystals shown in Fig. 4(c). This implies that nuclei were formed following irradiation and that crystals grew at a higher level of supersaturation at 5 °C, whereas no crystal grew at the lower level of supersaturation at 20 °C. The underlying mechanism remains unclear.

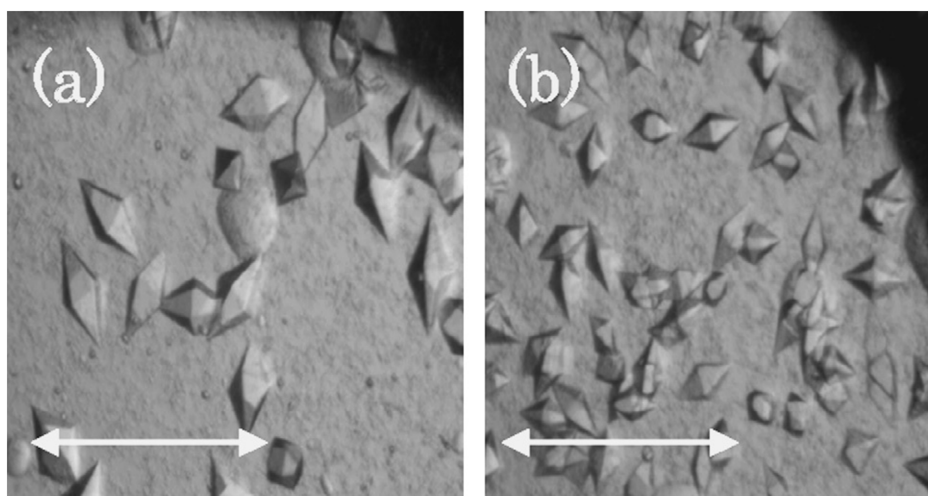

Fig. 3. (a) Photograph of a thaumatin droplet without irradiation. (b) Photograph of a thaumatin droplet with irradiation for 180 s. Lengths of the arrows, 300 μm.

Unstable small clusters were expected to be formed by irradiation. In the thaumatin system, since commercially available thaumatin contains some impurities, which induce undesired spontaneous nucleation, it is difficult to conduct an accurate and appropriate nucleation experiment to clarify the mechanism that operates in an undersaturated solution. For this purpose, we are currently investigating an ultrapure lysozyme system.

3.3. Photochemical mechanism

We will now discuss photochemical mechanism of light-induced nucleation. Steady-state electronic spectra and transient absorption measurements were carried out.

Fig. 5(a–e) shows the steady-state absorption and emission spectra of: (a) thaumatin, (b) lysozyme, (c) tryptophan, (d)

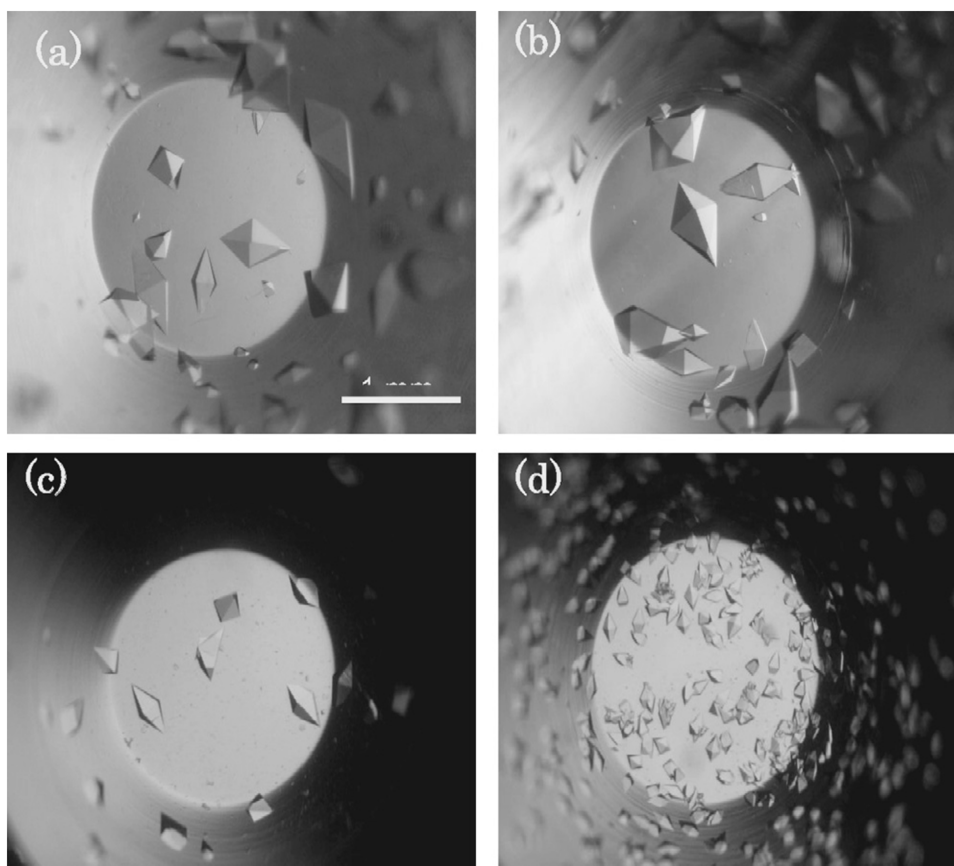

Fig. 4. Photograph of a thaumatin droplet. Undersaturated solutions ($\beta = 0.5$) were irradiated. The undersaturated solutions were mixed with concentrated thaumatin to induce the growth of nuclei for 24 h at 20 or 5 °C. (a) No irradiation (control experiment); (b) irradiation for 180 s followed by mixing of the solution and storage at 20 °C; (c) no irradiation followed by storage at 5 °C; (d) irradiation for 180 s followed by storage at 5 °C.

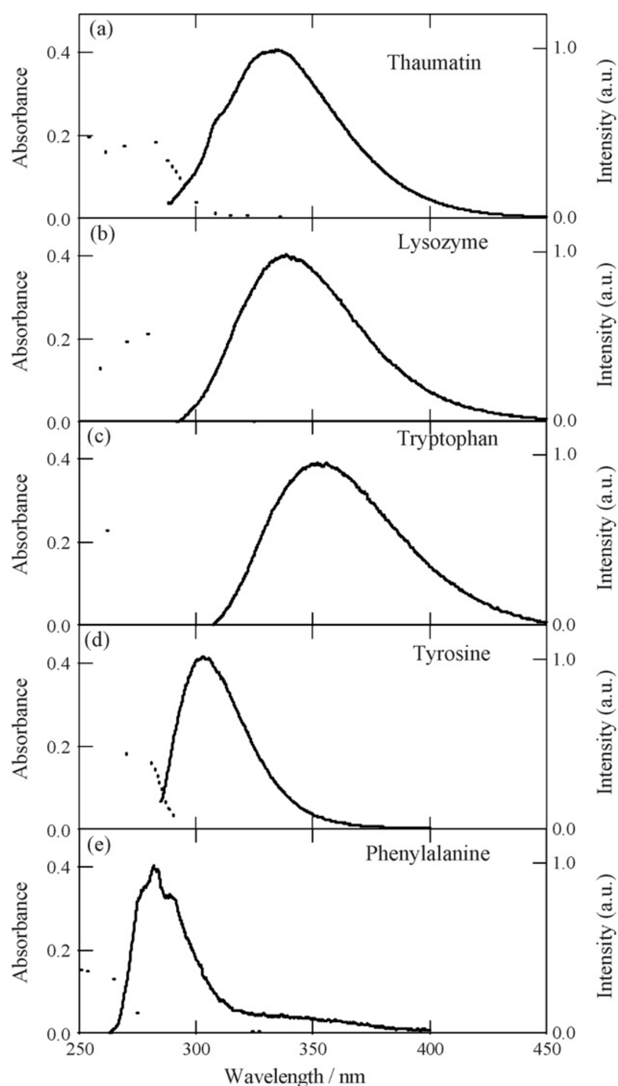

Fig. 5. Steady-state electronic spectra (absorption and emission) of: (a) thaumatin, (b) lysozyme, (c) tryptophan, (d) tyrosine, and (e) phenylalanine.

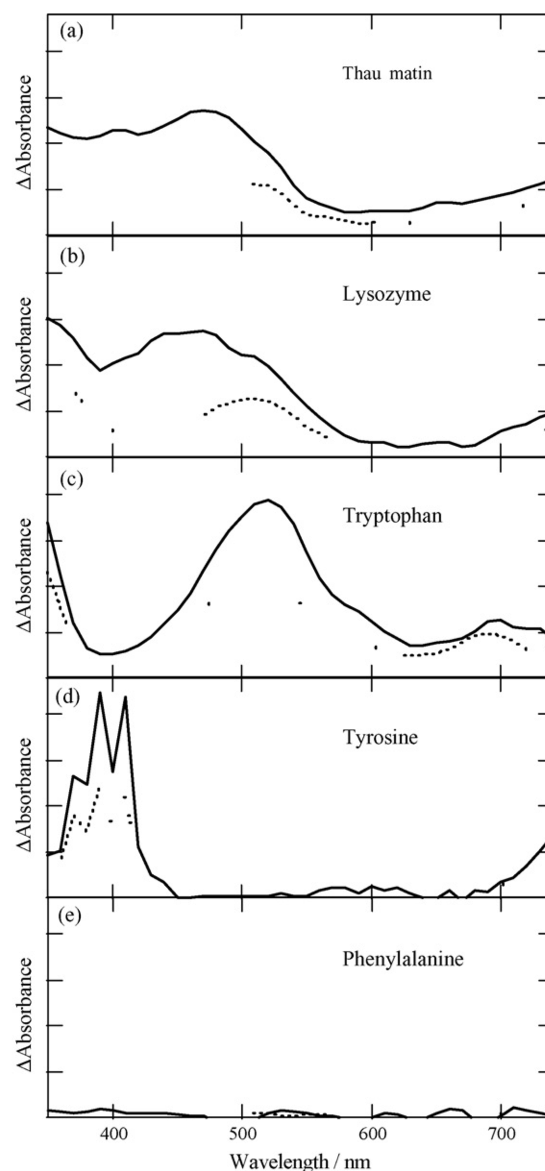

Fig. 6. Transient absorption spectra of: (a) thaumatin, (b) lysozyme, (c) tryptophan, (d) tyrosine, and (e) phenylalanine. The spectra were recorded 2 and 32 μs after laser excitation.

tyrosine, and (e) phenylalanine, respectively. Tryptophan, tyrosine and phenylalanine are amino acids that have π electron systems that can participate in photochemical reactions. Each of these amino acids emits fluorescence. The emission spectra of thaumatin and lysozyme are very similar to each other; these two emission spectra are almost identical to the emission spectrum of tryptophan and are different from the emission spectra of tyrosine and phenylalanine. Emission from lysozyme has been explained as intramolecular energy transfer [19]. In the lysozyme molecule, there are six tryptophan, three tyrosine, and two phenylalanine residues [20]. When one of these amino acids absorbs a photon, an excited-state amino acid is produced. The excited energy undergoes Förster-type intramolecular energy transfer. Förster-type energy transfer can take place between chromophores at a distance of 2–6 nm from each other [21]. Since the excited energy of tryptophan is the lowest of these three amino acids, only the excited state of tryptophan appears. Thus, the fluorescence of lysozyme can be attributed to the fluorescence from the tryptophan residues. Thaumatin has 3 tryptophan, 8 tyrosine, and 10 phenylalanine residues [16]. In the same manner, the emission spectrum of thaumatin is almost identical to the emission spectrum of tryptophan, and intramolecular energy transfer occurs in this system. Thus, it is reasonable to expect that the photochemical properties of thaumatin is analogous to that of lysozyme, i.e., the photochemical reaction of tryptophan.

Fig. 6 shows the transient absorption spectra of: (a) thaumatin, (b) lysozyme, (c) tryptophan, (d) tyrosine, and (e) phenylalanine in O_2-saturated solutions. Transient absorption spectra were observed 2 and 32 μs after pulsed laser excitation. The transient absorption spectra of thaumatin are, at a glance, very similar to the transient absorption spectra of lysozyme. Assignment of the transient species of lysozyme has been carried out previously. Triplet state is not detected because the sample solution was saturated with O_2 gas [22]. The spectra are composed of two different species. The fast decaying species

has absorption in the range of 350–600 nm and a lifetime of less than 4 μs. The fast decaying species was attributed to cystine electron adducts at the disulfide (–S–S–) bridge [23]. The other species has an absorption peak at 520 nm and a longer lifetime. The longer-lifetime transient species are identical to the spectrum of tryptophan (Fig. 6(c)) and differ from the transient species of tyrosine (d) or phenylalanine (e). Previous studies on lysozyme transient absorption assigned this intermediate species to a tryptophanyl radical [24,25]. In the present experiment, the same intermediate species was observed. Thus, the photochemical reaction of thaumatin is considered to be the photochemical reaction of tryptophan. This result strongly suggests that the photochemical intermediate induces the nucleation of thaumatin.

To confirm that the intermediate can form clusters, the chemical dynamics need to be considered. If the intermediate is responsible for nucleation, then the intermediate must encounter other molecules at least several times, so as to form nuclei with thaumatin within its lifetime. The mean time between two impacts, t, is calculated from the following equation: $t = d^2/(2D)$, where d is the root-mean-square distance between thaumatin molecules in solution, i.e., 12 nm at $C = 20\,\mathrm{mg\,mL^{-1}}$ ($\beta = 2$), and 19 nm at $C = 5\,\mathrm{mg\,mL^{-1}}$ ($\beta = 0.5$), and D is the diffusion coefficient of thaumatin. Since we do not know the diffusion coefficient of thaumatin, we use half the value of autodiffusion coefficient D_0 for lysozyme. The molecular mass of thaumatin is 22,000, which is 1.6 times larger than that of lysozyme ($D_0 = 10^{-10}\,\mathrm{m^{-2}\,s^{-1}}$ in water) [26]. The diffusion coefficient of thaumatin is assumed to be $D_0 = 0.5 \times 10^{-10}\,\mathrm{m^{-2}\,s^{-1}}$. This replacement value is probably overestimated. Based on the above assumption, the mean impact times are calculated as 2 μs for $\beta = 2$ and 4 μs for $\beta = 0.5$. This means that 250 collisions ($\beta = 0.5$) occur within the lifetime of the intermediate. If few collisions in 250 collisions generate cluster, the nuclei can grow to crystals in the supersaturated solution. For the transient absorption experiment, the root-mean-square distance was 41 nm and the mean impact time was 23 μs. Supersaturation of this solution could not be determined, as there was no precipitant in the sample solution. No nucleation took place in this solution. The mean impact time is comparable to the observation time.

In the present study, we confirm that thaumatin undergoes photochemically induced nucleation, and we demonstrate that the nucleation mechanism is analogous to that of lysozyme.

4. Conclusions

We report our second formal observations on photochemically induced nucleation of thaumatin crystals. The solubility of thaumatin was determined against potassium sodium tartrate (PST) at 20, 15, and 5 °C in ADA buffer. Photochemically induced nucleation was observed at supersaturation $\beta = 2$ at 20 °C. Moreover, light irradiation of an undersaturated solution with $\beta = 0.5$ temporally induced nucleation. The nuclei disappeared at 20 °C but grew at 5 °C. The temporal nuclei are considered to be unstable small clusters that are accumulated at the photochemical intermediate radical. These results suggest a new method for controlling crystal nucleation and growth, which may be useful for studies of structural genomics and pharmaceutical applications. In the future, we will study the interactions between irradiated species, which are expected to increase relative to those in a non-irradiated protein solution. The influence of light-irradiation on crystal quality will also be studied.

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