Tryptophan Neutral Radical Brings along Photochemical Crystallization

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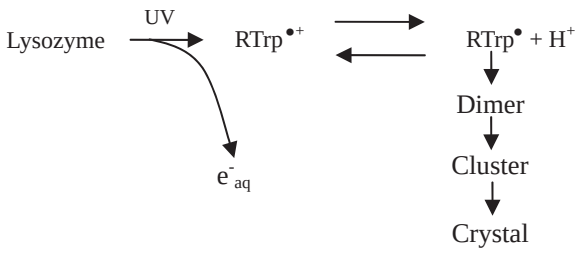
The mechanism of the photochemically induced nucleation of lysozyme was investigated. Radical cations and neutral radicals of tryptophan residue of lysozyme were observed by transient absorption measurements. The radical cation was found to be in equilibrium with the neutral radical, and the pK_a of the radical cation in UV–vis wide range buffer solution was estimated to be ca. 3.2. The dimer formation was confirmed by SDS-PAGE for the solutions with pH value higher than this pK_a value. The photochemically induced nucleation was also observed for these solutions. The number of crystals increased with increase of the dimer.

Crystallization of protein is important to reveal its 3D structure by means of X-ray diffraction crystallography. Recently, photophysically[1] and photochemically[2–6] induced nucleation of protein have been reported. In photochemically induced nucleation (PIN) of lysozyme, we found out that the number of crystals of lysozyme increased by UV-light irradiation and this increase depended on irradiation light-wavelength.[2] Neutral radicals of tryptophan residue of lysozyme (RTrp$^{\bullet}$) were observed in buffer solution at pH 4.3 by transient absorption measurements.[3] Photochemical dimerization of lysozyme was also observed by SDS-PAGE for this solution.[5] Based on these results, it was suggested that the dimer plays a role of the smallest cluster in the crystallization.[5,6] Scheme 1 shows the mechanism of PIN of lysozyme. Grossweiner et al. reported that the initial photochemical reaction of tryptophan residue of lysozyme is photoionization leading to the generation of radical cation (RTrp$^{\bullet+}$) and hydrated electron.[7] The RTrp$^{\bullet+}$ releases a proton to give the RTrp$^{\bullet}$.[8] The RTrp$^{\bullet+}$ is considered to be in equilibrium with RTrp$^{\bullet}$, since radical cations of tryptophan was found to be in equilibrium with the neutral radical. Therefore, quantity of RTrp$^{\bullet}$ should depend on the pH value of the lysozyme solution; if the pH value of the solution is higher than the pK_a value of RTrp$^{\bullet+}$, quantity of RTrp$^{\bullet}$ is large and the dimer formation may become efficient to enhance the nucleation. The pK_a value of the radical cation of tryptophan was reported to be ca. 4.3, but the pK_a value of RTrp$^{\bullet+}$ has not been reported yet.[9]

We, here, demonstrate the results of PIN experiments of lysozyme solution at several pH values. The pK_a value of RTrp$^{\bullet+}$ was estimated by analyzing transient absorption spectra observed at several pH conditions. SDS-PAGE was also carried out by employing solutions of pH values around the pK_a value.



Scheme 1. The mechanism of PIN of lysozyme.

Hen egg-white lysozyme was purchased from Seikagaku (6 times recrystallized lot E02Z04) and was used without further purification. The UV–vis wide range buffer (WR buffer) was prepared and used as a solvent.[10] The pH of solvent was adjusted by adding HCl or NaOH aqueous solution. All reagents (GR-grade) used for solvent preparation were purchased from Wako Pure Chemical Ind. Ltd. Experimental setup for transient absorption measurements were described in a previous paper.[2] Sample solutions were flowed at a flow rate of 40 mL min^{-1} through a sample cavity cell and pumped by 266-nm laser flash (1 mJ cm^{-2} pulse^{-1}).[2] SDS-PAGE experiment was described in a previous paper.[5] Liquid-seeding was used for the crystallization experiment in 72-well microbatch plates, purchased from Hampton Research.[3] Sample droplets were mixed with NaCl solution and then dropped in the well covered with paraffin oil. The plate was kept in an incubator at 22 °C for 1 week. UV-light irradiation was carried out by use of 300-W Xe lamp (USHIO, UXL-300SX) filtered by band pass filter (SIGMA KOKI, UVTF-33U).[5]

To estimate the pK_a value of RTrp$^{\bullet+}$, transient absorption spectra of lysozyme were measured by nanosecond laser flash photolysis. Figure 1 shows transient absorption spectra of lysozyme in WR buffer solution at pH 2.0 (a), 3.0 (b), 4.3 (c), and 5.0 (d). These spectra were recoded at 64 μs after the laser flash. Figure 1a shows an absorption band in the region of 500–650 nm, whereas Figures 1c and 1d show an absorption band in the region of 450–600 nm. The main intermediates

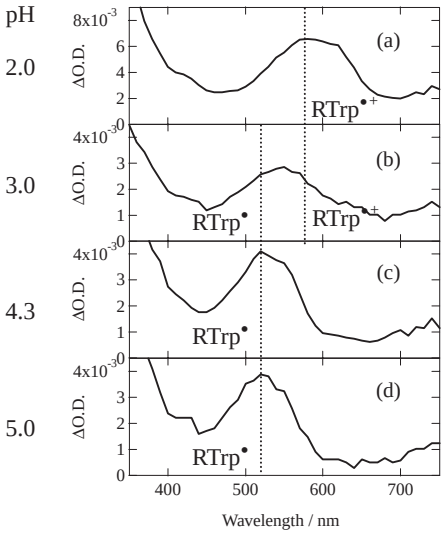


Figure 1. Transient absorption spectra of lysozyme in WR buffer solution at pH 2.0 (a), 3.0 (b), 4.3 (c), and 5.0 (d). These spectra were recorded at 64 μs after the 266-nm laser flash.

of lysozyme observed are $RTrp^{\bullet+}$ ($\lambda_{max} = 570$ nm), $RTrp^{\bullet}$ ($\lambda_{max} = 520$ nm), and hydrated electron ($\lambda_{max} > 650$ nm).[7] While, in Figure 1b an absorption band was observed in the region between those of (a) and (d). The solutions at pH 6.0, 8.0, 10.0, and 12.0 show essentially the same spectra as that of (d) (these spectra are not shown). Transient absorption spectra of lysozyme are similar to those produced from tryptophan upon the photolysis.[7–10] The main absorption species at pH 2.0 and 4.3–5.0 are ascribed to $RTrp^{\bullet+}$ and $RTrp^{\bullet}$, respectively. As a result, the pK_a value of $RTrp^{\bullet}$ of lysozyme is estimated to be ca. 3.2. Thus, the pK_a value of $RTrp^{\bullet+}$ is confirmed to be a little small compared with that of the radical cation of tryptophan.

To examine the pH dependence of quantity of dimer generated by UV-light irradiation, SDS-PAGE were carried out for samples at pH 2.0, 3.0, 4.3, and 5.0, and the photograph of SDS-PAGE gel is shown in Figure 2. Lane 1 is the molecular weight marker. Lanes 2–5 are samples without light irradiation. Lanes 6–9 are samples irradiated for 30 min. The acidity of each lane was indicated as pH value shown below the lane numbers. The lysozyme monomer band at 14 kDa was observed in Lanes 2–9. The lysozyme dimer band at 28 kDa was observed for the samples irradiated for 30 min at pH 4.3 and 5.0 as shown in Lanes 8 and 9, respectively. The intensity of lysozyme dimer band increased with increase in acidity of sample solutions.

To confirm enhancement effects of the dimer on lysozyme nucleation, crystallization experiments were carried out for the samples at different pH values. Three pH values (pH 2.0, 4.3, and 5.0) were selected to control the quantity of the dimer. Figure 3 shows photographs of PIN of lysozyme at different pH values: (a), (b) at pH 2.0, (c), (d) at pH 4.3, (e), (f) at pH 5.0. Irradiation time was 0 or 120 s. The lysozyme concentration of all droplets was adjusted at 12.5 mg mL^{-1} in 0.6 M NaCl to avoid spontaneous nucleation at pH 4.3. In all droplets at pH 2.0, five crystals were observed as shown in Figures 3a and 3b, indicating that crystallization at pH 2.0 is independent of the irradiation time. These results suggest that PIN did not take place at pH 2.0. Since lysozyme has an isoelectric point (pI) at about 11,[11] the solubility decreases when the pH of solution is close to pI. The solution of pH 2.0 has the highest solubility in this experiment and the crystallization of lysozyme is generally considered to be the most difficult. It is known that extremely low or high pH condition induces denaturation of proteins. In the solution at pH 2.0, denatured lysozyme is generally considered to decrease the solubility and consequently increase the supersaturation level enough to nucleate. In droplets without irradiation at pH 4.3 (c) and 5.0 (e), no crystal was observed,

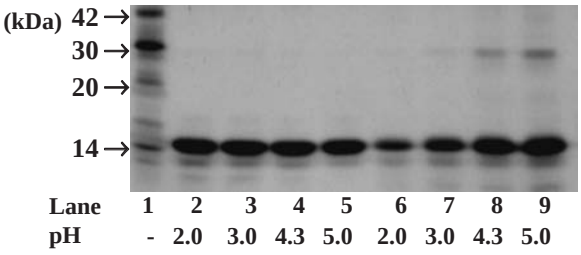


Figure 2. Photograph of gel. Lane 1: molecular weight marker, Lanes 2–5: lysozyme solution without irradiation, Lanes 6–9: lysozyme solution with UV-light irradiation for 30 min. The acidity of sample solution is indicated below the lane numbers.

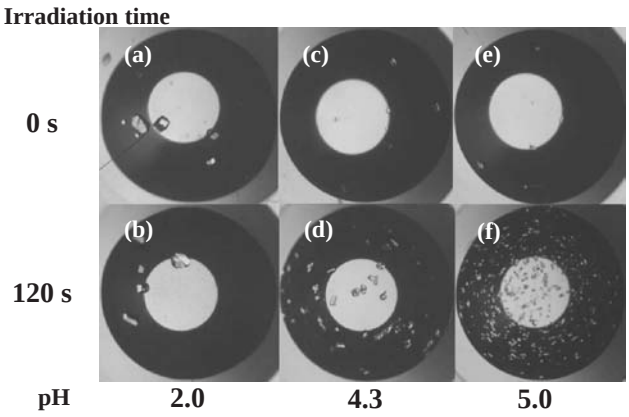


Figure 3. Photographs of PIN of lysozyme at different pH value: (a), (b) at pH 2.0, (c), (d) at pH 4.3, (e), (f) at pH 5.0. Irradiation time was 0 and 120 s. The lysozyme concentration of all droplets was adjusted at 12.5 mg mL^{-1} in 0.6 M NaCl solution.

while in the irradiated droplets (d) and (f), crystals were appeared. The number of crystals in each droplet was 25 and 75 for (d) and (f), respectively. The number of crystals observed at pH 5.0 (f) is 3 times larger than that at pH 4.3 (d). Based on the pK_a value of $RTrp^{\bullet+}$, the quantity of $RTrp^{\bullet}$ at pH 5.0 is estimated to be approximately 5 times greater than that at pH 4.3. If all of the $RTrp^{\bullet}$ combine to give the dimer, the quantity of dimer at pH 5.0 would be 2.5 times greater than that at pH 4.3. These consideration lead to conclusion that the dimer enhances lysozyme crystallization when $RTrp^{\bullet}$ is generated in the solution with pH value higher than pK_a value of $RTrp^{\bullet+}$.

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