Detection of Covalent-bonded Dimer in Photochemically Induced Crystallization of Protein

Kenji Furuta,[1] Tetsuo Okutsu,*[1] Gen Sazaki,[2] Izumi Yoshizaki,[3] Hiroaki Horiuchi,[1] Tetsuya Shimizu,[4] Masaki Yamamoto,[4] Yoshihito Tanaka,[4] and Hiroshi Hiratsuka[1]
[1]*Department of Chemistry, Gunma University, Kiryu 376-8515*
[2]*Institute for Materials Research, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577*
[3]*Japan Aerospace Exploration Agency, 2-1-1 Sengen, Tsukuba 305-8505*
[4]*RIKEN SPring-8 Center, 1-1-1 Kouto, Sayo 679-5148*

(Received February 21, 2007; CL-070197; E-mail: okutsu@chem.gunma-u.ac.jp)

We investigate light-induced crystallization mechanism of hen egg-white lysozyme. Photochemical intermediate radical has been expected to form cluster which grows to nucleus. To confirm the nucleation process that the radical grows to the crystal, SDS-PAGE, crystallization experiments were carried out. Covalent-bonded dimer was produced by photochemical reaction, and it grows to the crystal. We conclude that the dimer plays the role of the smallest stable cluster in early stage of the crystallization process.

Crystallization of protein is important to reveal its 3D structure by X-ray diffraction crystallography. Recently, photophysical or photochemical light-induced crystallization of protein has been reported.[1–4] In our preliminary studies, we demonstrated photochemically induced nucleation of hen egg-white lysozyme in metastable solution.[3,4] We irradiated UV-light from a Xe-lamp to metastable solutions. Several crystals appeared only in the irradiated solution. We have investigated photochemical mechanism of the light-induced crystallization of lysozyme and analogous proteins.[5] The mechanism is illustrated in Figure 1; photochemical intermediate, residual tryptophanyl radical was produced by one-photon absorption. When the intermediate was denatured by second photon, light-induced nucleation was inhibited. Therefore, the intermediate was concluded to grow to nucleus. However, we do not know how the intermediate grows to crystal.

In a preliminary paper, we reported that addition of polyethylene glycol 4000 (PEG4000) to lysozyme solution greatly enhanced the frequency of light-induced crystallization.[6] Addition of PEG4000 affects to enlarge reaction rate constant between intermediate radicals. The intermediate is expected to form protein dimer.

We, here, report the detection of photochemical lysozyme dimer by SDS-PAGE. We confirmed that the dimer grows to nucleus by crystallization experiment.

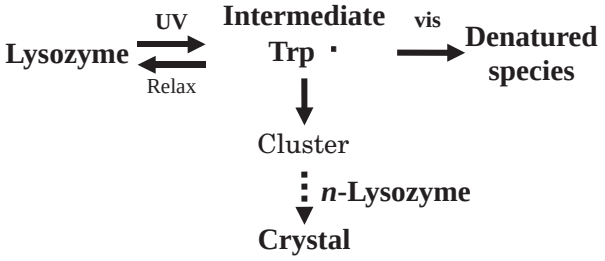


Figure 1. Mechanism of photochemically induced nucleation of lysozyme.

Hen egg-white lysozyme was purchased from Seikagaku (six times recrystallized, lot E02204) and used without further purification. Lysozyme solution was prepared at 1 mg·mL^{-1} concentration in 50 mM, pH 4.3 sodium acetate buffer. Prepared solution was centrifuged at 10,000 rpm for 5 min. Then the solution was filtered by 0.45 μm membrane filter (NALGENE). Light irradiation was carried out as described in literatures.[3,4] As a control sample lysozyme dimer was used.[7] Five microliters of sample solutions was loaded onto each lane of the gel. The gel was stained by silver stain kit (Wako).

Figure 2 shows a photograph of gel. Lanes 1–4 are lysozyme solution containing NaCl (0.1 M), lane 5 is lysozyme dimer, lanes 6–9 are lysozyme solution without NaCl and lane 10 is molecular weight marker from 14 to 79 kDa. These sample solutions were irradiated by UV-light for 0, 15, 30, and 60 min. Irradiation times are indicated under the lane numbers. The commercially available lysozyme contains dimer at 28 kDa (0.5%), unknown impurity at 18 kDa (1.0%) and small amount of other impurities smaller than 14 kDa.[8] Lane 1 is the solution without irradiation which indicates mainly lysozyme monomer at 14 kDa and smaller weight bands. The dimer and 18 kDa impurity bands were not detected in this experimental condition. Lanes 2–4 show irradiated samples. The dimer band

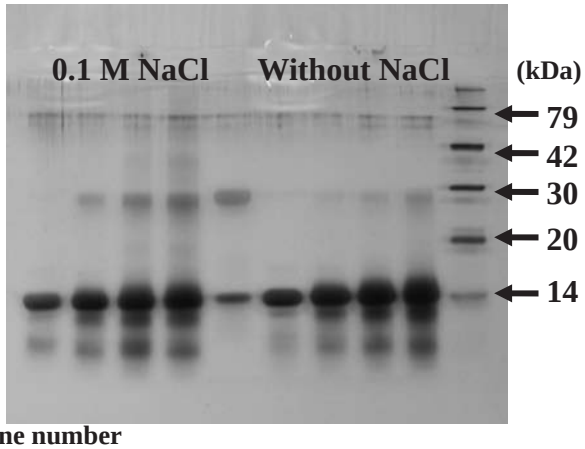


Figure 2. Photograph of gel. Lanes 1–4: lysozyme solution contains NaCl (0.1 M), lane 5: lysozyme dimer, lanes 6–9: lysozyme solution without NaCl, lane 10: molecular weight marker. These sample solutions were irradiated by UV light for 0, 15, 30, and 60 min. Irradiation times are indicated below lane numbers.

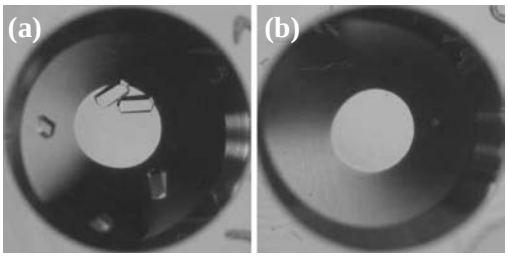


Figure 3. Photographs of droplets of lysozyme solution. These droplets contain (a) dimer and fragments and (b) only fragments. The diameter of circle is 1 mm.

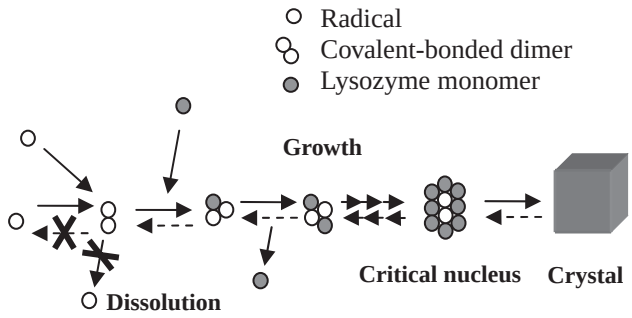
and the smaller weight band intensity were increased with increasing in irradiation time.

Samples without NaCl were also loaded onto the same gel. Lanes 6–9 show irradiated samples. In this case, smaller band intensity was increased but the dimer band intensity was hardly increased by the irradiation. Since, NaCl is known to take place salting-out which reduces electrostatic repulsion between charged lysozyme (+11 at pH 4.5), covalent-bonded dimer formation becomes to happen in the presence of NaCl.

By the irradiation, dimer and small fragments were produced. Then, we discuss which species are responsible for the nucleation. To clarify the dimer or fragments grow to nucleus, crystallization experiment was carried out. We prepared two solutions, one was lysozyme solution which contains 0.7 M NaCl (solution A), and the other solution was lysozyme solution without NaCl (solution B). Then, these solutions were irradiated for 60 s. As expected in the SDS-PAGE experiment, photochemical dimer exists only in the solution containing NaCl. Small weight fragments exist in both solutions. After the irradiation to the solution B, NaCl was added. NaCl concentration was equally adjusted to solution A. These solutions were kept for 1 week at 22 °C. The results are shown in Figure 3. Figure 3a is lysozyme solution (A) which contains dimer and fragments (irradiated solution with NaCl). Several crystals appeared. Figure 3b is lysozyme solution (B) which did not contain dimer (irradiated solution without NaCl). No crystal appeared. These results conclude that the fragments do not grow to the crystal but the dimer grows to the crystal.

As a conclusion, photochemically induced nucleation mechanism is explained. Nucleation mechanism is illustrated in Scheme 1. Molecules gather to form cluster $n = 2, 3, 4, \ldots$ to bulk crystal. When the cluster size is small, clusters are unstable owing to surface/volume energy disadvantage. Growth and dissolution take place even in supersaturation. After the cluster size becomes larger than the critical size, the cluster grows to bulk crystal spontaneously. Critical size of the nucleus depends on supersaturation, small at high supersaturation and large at low supersaturation.[9] In our experimental condition, critical nucleus size is assumed to be $n \approx 9$–10.[9,10]

The first step of normal nucleation process begins from the formation of $n = 2$ smallest cluster. The smallest cluster bound by weak interaction (van der Waals or hydrogen bond)



Scheme 1. Light-induced nucleation mechanism of protein. Photochemical product, protein dimer, behaves as smallest cluster. The photochemical dimer is stable and the nucleation is accelerated.

is unstable. Thus, the $n = 2$ cluster formation is important step among the early stage of nucleation process. Whereas, if the photochemical covalent-bonded dimer behaves similarly to $n = 2$ cluster, the dimer grows to $n = 3, 4, \ldots$ to critical size. Therefore, nucleation starts from stable cluster, i.e., photochemical dimer, nucleation frequency should be higher.

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