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Invited paper

Photo sensitization reaction-induced crystallization of lysozyme

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ABSTRACT

The photochemical reaction of triplet benzophenone (^{3}BP) induces the crystallization of hen egg-white lysozyme by producing a BP ketyl radical and a counter amino acid radical in lysozyme, resulting in lysozyme dimerization.

Crystallization was induced by irradiation of a BP/lysozyme metastable solution. The 20 amino acids were screened to determine which react with ^{3}BP, and tryptophan (Trp), tyrosine (Tyr), and methionine (Met) were identified as reactive. The fluorescence of the Tyr–Tyr chromophore increased with increasing irradiation time and simultaneously Trp fluorescence decreased. Therefore, ^{3}BP first reacts with a Trp residue in lysozyme to form a Tyr–Tyr covalently bound dimer that acts as a crystallization nucleus.

1. Introduction

Determination of the three-dimensional structure of a protein is carried out by X-ray crystallography, and the protein structure allows researchers to design molecules to control the function of that protein, furthering drug discovery [1–3].

Studies on the photo-induced crystallization of proteins have recently been conducted [4–7]. Light and protein molecules interact physically and this interaction can be used to crystallize proteins. Examples of the photo-induced crystallization of protein include laser ablation with pulsed laser light to concentrate protein molecules and promote nucleation [8–10], and the physical focusing of protein molecules at a focal point using the strong electric field of condensed laser light to grow crystals [11–13].

We previously reported a photochemical reaction in which lysozyme crystallization is accelerated when light is irradiated onto a metastable solution of the protein [14–18]. We measured the transient absorption spectra of lysozyme and showed that the reaction intermediate radical of a tryptophan (Trp) residue is involved in this crystallization process. A protein dimer is formed in solution during irradiation and this dimer grows to form a nucleus. Changing the pH of the solution controlled the intermediate species of the Trp radical, revealing that the dimer is formed by generation of a neutral radical Trp residue [19]. The dimer forms a covalent bond between two Tyr residues (Tyr 53) in the two lysozyme molecules. For the dimer to act as a template and grow into a crystal, the two covalently bound protein molecules must adopt a structure similar to that of two adjacent molecules in a lysozyme crystal [20,21].

In the present study, we investigated a method for reacting amino acids on the surfaces of neighboring lysozyme molecules using a hydrogen atom abstraction reaction. The excited triplet state of benzophenone is used as a photosensitizer to produce protein dimers, and amino acids other than Trp and Tyr are radicalized. The structure of the reaction product acts as a template for lysozyme crystallization.

2. Experimental

Hen egg-white lysozyme was purchased from Seikagaku (6 times recrystallized, lot E02Z04) and was used without further purification. Benzophenone (BP) was GR-grade and was used as photosensitizer. Sodium acetate, acetic acid, L-tryptophan (Trp), L-tyrosine (Tyr), L-methionine (Met), L-phenylalanine (Phe) sodium chloride, and ammonium sulfate were GR-grade and were purchased from Wako Pure Chemicals.

L-Alanine (reagent grade), L-valine (cell culture tested), L-leucine (cell culture tested), L-isoleucine (reagent grade, ≥98%), L-proline (ReagantPlus™, ≥99%), L-methionine (reagent grade, ≥98%), L-serine (ReagantPlus™, ≥99%), L-threonine (reagent grade, ≥98%), L-cysteine (cell culture tested), L-asparagine (≥98%), L-glutamine (cell culture tested), L-aspartic acid (≥98%), L-glutamic acid (cell culture tested), L-arginine (reagent grade, ≥98%), L-lysine

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(≥98%), and L-histidine (ReagentPlus™, ≥98%) were purchased from Sigma Aldrich Chemical.

Sodium acetate and acetic acid were dissolved in ultra-pure water (Milli-Q) and used as the buffer solution (NaAc buffer, 50 mM, pH 5.5). The buffer was centrifuged and filtered through a 0.45-μm membrane filter (NALGENE) prior to crystallization experiments. Sample preparation was carried out at room temperature.

Steady-state spectra were recorded using a HITACHI F4500 fluorescence spectrometer for emission measurements and a HITACHI U3300 spectrometer for absorption measurements. Transient absorption spectra were measured using a Nd^{3+}: YAG laser (Lotis Tii LS-2137U, 355 nm, 30 ns HWFM, 3 mJ pulse^{-1}, 10 Hz) as the excitation light source. The sample solutions were flowed through a quartz cavity cell at a flow rate of 40 ml min^{-1} and the transient signals were detected using a photomultiplier tube. The output signals were measured by a digital oscilloscope (Tektronix TDS 380P) and transferred to a personal computer. Sample solution used in transient absorption experiment was bubbled by Ar gas for 15 min to purge oxygen gas. A Nd^{3+}: YAG laser was used to irradiate samples and to photochemically induce crystallization. SDS-PAGE was conducted using a slab minigel electrophoresis unit (Nihon Eido, NA-1020, CN-1010) and 15% polyacrylamide resolving gels with 0.05% polyacrylamide stacking gels. Tris-Gly buffer solution (containing 0.4% SDS) was used as the electrode solution. Sample solution (4 μl) was run in each lane of the gel and the developed gel was stained using a silver stain kit.

The batch crystallization experiment was carried out in a 72-well micro batch plate purchased from Hampton Research. The plate was covered with paraffin oil, then the protein droplets were added. The droplets were irradiated for 300 s with 355 nm laser light through the paraffin oil, then the plate was sealed with silicone grease and stored at 20 °C for one day.

3. Results and discussion

Fig. 1 shows the absorption spectra of benzophenone (BP) and lysozyme in buffer solution. Lysozyme absorbs below 310 nm whereas BP absorbs at longer wavelengths (up to 380 nm). BP could therefore be excited with 355 nm light without exciting lysozyme. We determined whether BP is dissolved in the aqueous phase or adsorbed onto lysozyme by adding a crystallization agent (NaCl) to the BP(1.0×10^{-3} M)/lysozyme(10 mg mL^{-1}) system to precipitate the lysozyme. No BP absorption was observed in the supernatant, indicating that BP is adsorbed onto lysozyme molecules in water.

Fig. 2 shows a SDS-PAGE gel with four lysozyme samples: with/without irradiation with 355 nm YAG laser light and with/without BP. Lane 1 is the control (no irradiation, no BP) and shows a lysozyme monomer band and a faint dimer impurity. Lane 2 is the irradiated sample without BP. Lane 3 is the non-irradiated lysozyme sample in the presence of BP. The results of lane 2 and lane 3 are identical to the control. In contrast, lane 4 shows a clear lysozyme dimer band at 28 kDa. We previously demonstrated the photochemical induction of protein crystallization via formation of a dimer that grows to form a nucleus.

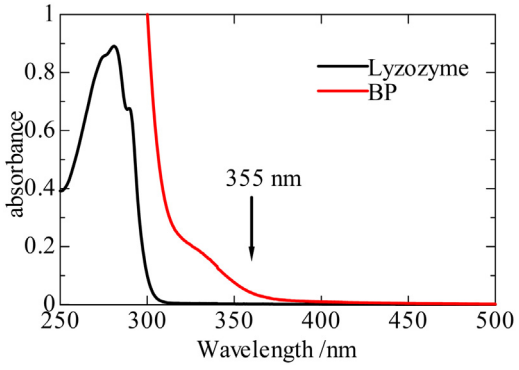

Fig. 1. Absorption spectra of lysozyme (black) and benzophenone (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

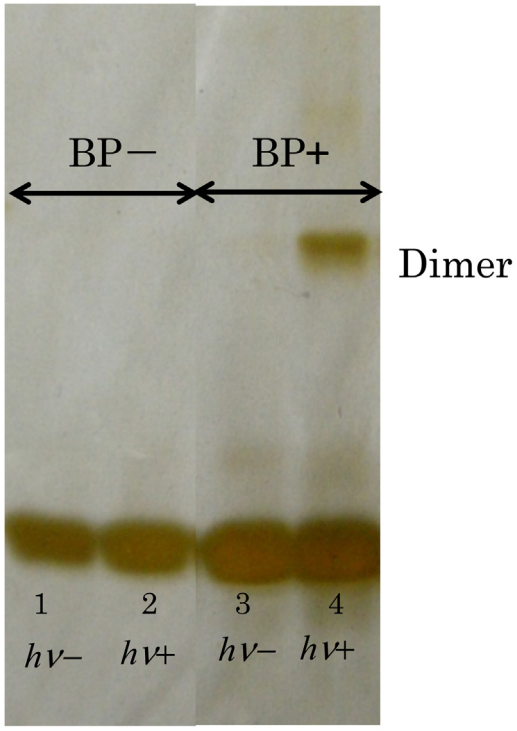

Fig. 2. SDS-PAGE of photo-irradiated lysozyme without/with BP.

Our present aim was to induce lysozyme crystallization by photochemical perturbation in the absence of spontaneous nucleation. A metastable supersaturated solution was prepared using 10 mg mL^{-1} lysozyme and 0.7 M NaCl as precipitant. The supersaturation was 3. Fig. 3 shows photographs of 2 × 2 matrixes with/without irradiation and with/without BP. The control experiment (BP−, hν −) shows no crystals after 24 h and only

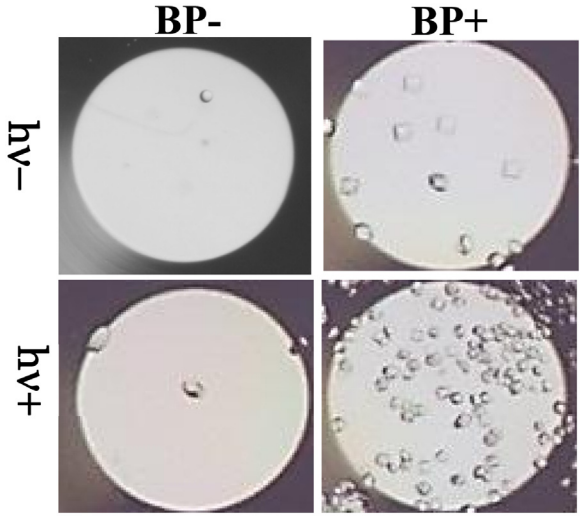

Fig. 3. Photographs of protein solutions without/with light irradiation and without/with BP.