Mechanism of the photochemically induced nucleation of proteins

THESIS

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DOCTOR OF ENGINEERING

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## Contents

I. General Introduction 1
   I-1 Structure-based drug design 2
   I-2 Major techniques for structure solution: crystallization 3
   I-3 Crystallization theory 4
   I-4 Progress of protein crystallization techniques by use of external perturbation 6
   I-5 References 7

II. Photochemically induced nucleation of Hen egg-white lysozyme 19
   II-1 Introduction
      II-1-1 Hen egg-white lysozyme 20
      II-1-2 Previous works of light induced nucleation of lysozyme 20
   II-2 Experimental section
      II-2-1 Sample preparation and experimental apparatus 24
      II-2-2 Light irradiation 24
      II-2-3 Crystallization experiment 25
      II-2-4 Transient absorption measurements 25
      II-2-5 Enzymatic activity measurements by the ML method 26
      II-2-6 SDS-PAGE experiment 26
   II-3 Results and Discussion
      II-3-1 Electron spectrum measurements 27
      II-3-2 Slide glass tests 27
      II-3-3 Development of a crystallization technique 28
      II-3-4 Transient absorption measurements 30
      II-3-5 Enzymatic activity measurements 32
II-3-6 Crystallization by two-color light-irradiation

II-3-7 Dimer detection by SDS-PAGE experiment

II-4 Summary

II-5 References

III. pH dependence of photochemically induced nucleation of lysozyme

III-1 Introduction

III-2 Experimental section

III-3 Results and Discussion
   III-3-1 Transient absorption measurements
   III-3-2 Dimer detection by SDS-PAGE experiments
   III-3-3 pH dependence of photochemically induced nucleation of lysozyme

III-4 Summary

III-5 References

IV. Photochemically induced nucleation of Bovine pancreatic Ribonuclease A

IV-1 Introduction

IV-2 Experimental section
   IV-2-1 Sample preparation
   IV-2-2 Crystallization experiment and UV irradiation apparatus

IV-3 Results and Discussion
   IV-3-1 Electronic spectrum measurements
   IV-3-2 Transient absorption measurements
   IV-3-3 Dimer detection by SDS-PAGE experiment

IV-3-4 Photochemically induced nucleation of Bovine pancreatic Ribonuclease A
| IV-4 Summary                        | 88 |
| IV-5 References                    | 89 |
| V. Conclusion                      | 103 |

*Acknowledgements*                  106
Chapter I

General Introduction
I General introduction

I-1 Structure-based drug design

Protein X-ray crystallography is one of the premier techniques for elucidating three dimensional structures at atomic resolution [I-1, 2, 3]. Crystal structures provide the exact nature of protein-ligand interactions and catalytic mechanism of enzymes. Information about the structural, chemical and dynamic landscapes of a macromolecular drug target has been used for the development of small-molecule drugs through a process termed structure-based drug design (SBDD) [I-4]. SBDD is an iterative method that combines protein structure and molecular modeling to develop and optimize small-molecular inhibitors. By the determination of crystal structures of protein targets with substrates, natural product inhibitors, compounds derived from libraries or anew-designed scaffolds, medicinal chemists and molecular modelers are able to devise strategies for making rational modification to molecules that may improve compound affinity and selectivity. Scheme I-1 shows flowchart of structure-based drug design. After the first set of protein-ligand structures is determined, further its active site of rational drug candidates is design and modification, target structure-based screening and cocrystal structure determination are used to develop potential drug candidates. SBDD has the potential to accelerate the rate at which small-molecule candidates reach the clinic by reducing the number of compounds that have to be synthesized and tested.

Although the ability to effectively act upon the drug target is an essential feature of drug candidates, their utility ultimately depends on additional properties that are not directly assessed in most SBDD regimens. However, by precisely localizing regions of the small molecules involved in the key protein-ligand interactions, SBDD provides insights into how modifications to the compound can be made without compromising ligand affinity. Thus, information from testing several small-molecule candidates in animals, or in surrogate tests when available, can provide insights on how to rationally design drug candidates with
improved pharmacokinetic properties or a reduced toxicity profiles. Table I-1 shows drugs derived from structure-based approaches. Several drugs currently on the market such as the HIV protease [I-5] inhibitors Agenerase\textsuperscript{TM} (Vertex and GlaxoSmithKline) [I-6], Viracept\textsuperscript{TM} (Agouron) [I-7], neuraminidase [I-8] inhibitors Relenza\textsuperscript{TM} (GlaxoSmithKline) [I-9] and Tamiflu\textsuperscript{TM} (Gilead Sciences and Roche) [I-10] were developed through structure-based methodologies.

**I-2 Major techniques for structure solution: crystallization**

Protein crystallography has been progressing slowly, because protein crystallization is labor intensive due to a number of bottlenecks.

Protein structures are stored in a global responsibility called the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) which, from its inception 1971, has grown from less than a dozen structures to over 47500 structures in 2007; many of which are complexes that contain other relevant molecules and ligands such as drugs or co-factors. The first protein structure (Myoglobin) was determined by Kendrew et al. in 1960s by use of X-ray crystallography [I-11]. Since then, structures of many other proteins have been determined using mostly X-ray crystallography but also nuclear magnetic resonance (NMR) spectroscopy. Table I-2 shows methods for the structure determination and number of proteins listed in PDB. Liganded X-ray structures provide a wealth of information that can significantly assist and guide the drug development process.

Historically, the 3D protein structure determination requires 1-20 years for individual protein or protein assembly. Scheme I-2 shows the structure solution procedure using X-ray crystallography, consisting of cloning, expression, solubilization, purification, crystallization and diffraction steps. The bottleneck of some protein samples was reduced with the molecular biology tools developed in the 1980s and 1990s; currently exist tools are parallel expression
and purification of a number of gene products [I-12 - 16]. This approach enables the exploration of multiple constructs, homologs and variants for specific protein targets. The second major advance has been in the structure determination process. For example, the use of multiple-wavelength anomalous dispersion (MAD) using selenomethionine incorporated into the overexpressed protein [I-17, 18], diffraction data collection at synchrotron beam-lines using flash cooling [I-19], beam-lines robotics and automated structure solution methods [I-20 - 22] have all provided tremendous breakthroughs enabling the high-throughput structural biology.

Figure I-1 shows the proportion of success on each experimental step. These data were cited from statistical results of structure solution projects; Berkeley Structural Genomics Center (BSGC, circle), Midwest Center for Structural Genomics (MCSG, triangle) and Southeast Collaboratory Structural Genomics (SECSG, square). It is understood that crystallization remains one of the rate-determining steps to solve macromolecular structure.

I-3 Crystallization theory

The technique of protein crystallization is an underdeveloped subject, and a trial and error procedure has been mainly employed for the crystallization of each protein. The birth of a nucleus, the so-called nucleation process, is one of the most fascinating problems in protein crystallization. This nucleus absorbs growth-units from supersaturated environment to grow to crystal, so called crystal growth process [I-23].

Figure I-2 shows a typical solubility curve of protein vs. the salt concentration. A solution whose composition lies below the solubility curve is “undersaturated”. In this undersaturated region, crystal dissolves into solution, while in the “supersaturated” region proteins nucleate and grow to crystal [I-24, 25]. Therefore, production of a supersaturated solution is a prerequisite for crystallization. Formally the supersaturation, $\beta$, is defined as the ratio between
the protein concentrations in an equilibrium state, \( C_{eq} \), and in supersaturated state, \( C_{ss} \):

\[
\beta = \frac{C_{ss}}{C_{eq}}
\]

To obtain precise X-ray crystallographic data it is necessary to prepare a single crystal from a solution of low supersaturation level, because a solution of higher supersaturation level gives only amorphous precipitation. Here, I discuss the nucleation and growth of crystal from the point of view of thermodynamics. Figure I-3 shows the free energy change (\( \Delta G \)) as a function of critical cluster radius \( (r) \) in nucleation. Free energy, \( \Delta G \), is defined in terms of surface energy disadvantage \( (4\pi r^2 \gamma) \) and volume advantage \( (-4\pi r^3 \ln \beta / 3V) \):

\[
\Delta G = 4\pi r^2 \gamma - 4\pi r^3 kT \ln \beta / 3V
\]

where \( r \) is cluster radius, \( \gamma \) is surface tension, \( k \) is Boltzmann constant, \( T \) is Kelvin temperature, \( \beta \) is supersaturation and \( V \) is cluster volume. The critical cluster radius \( r^* \) at the maximum \( (\Delta G^*) \) in free energy corresponds to the size at which further growth of the cluster leads to a decrease in free energy. For a cluster with radius less than \( r^* \) the decrease in free energy can only be achieved by dissolution. At the same time, \( \Delta G^* \) is considered to be activation barrier at critical cluster radius \( r^* \). Figure I-4 shows variation in the activation barrier as a function of \( \beta \). At high \( \beta \), when the radius of the critical cluster becomes small, the activation barrier disappears and amorphous forms. Thus, an energy barrier with a proper height is necessary to obtain a single crystal.

The crystallization process is summarized as shown in Figure I-5. Molecules gather to form clusters consisting of 2, 3, 4, molecules and finally grow to bulk crystal. When the size is small, clusters are unstable, due to the surface/volume energy disadvantage and grow or dissolve even under supersaturation. When the cluster size becomes larger than the critical size, the nucleus grows to bulk crystal.

In the presence of impurities, nucleation often occurs in protein solution, called “heterogeneous nucleation”. Foreign bodies can induce nucleation at a supersaturation level
lower than that required for “homogenous nucleation”. A catalytic surface also induces nucleation by the adsorption of crystallizing materials to reduce the value of $\Delta G^*$. The extent of this reduction depends on the degree to which the catalyzing body mimics the structure of the crystallizing material.

To obtain high quality crystallographic samples, we have to set up an optimum crystallization condition; the nucleus should be formed at a level of supersaturation lower than amorphous area and higher than solubility. For an efficient crystal growth, supersaturation must be reduced to a lower level; a high supersaturation would result in the formation of too many nuclei and then too many small crystals.

I-4 Progress of the protein crystallization technique by use of external perturbation

Novel nucleation techniques of protein have been recently reported by physical chemists and crystallographers. Most of these techniques are making use of external perturbation such as cooling [I-26], electric field [I-27 - 29], magnetic field [I-30 - 34], micro-gravity [I-34, 35], femto-second laser pulse [I-36, 37] and ultra-sounds radiation [I-38, 39].

In this study, I employed photochemical reaction to induce protein nucleation in low supersaturated solution where spontaneous nucleation does not take place. Hen egg-white lysozyme and bovine pancreatic ribonuclease A were selected as model proteins, and as the first step, development of the crystallization technique making use of photochemical reaction of lysozyme was investigated. To clarify the nucleation mechanism, transient absorption measurements, enzymatic activity measurements and SDS-PAGE were carried out and the mechanism was deduced from the experimental results. As the second step, for further confirmation of the mechanism, crystallization of lysozyme has been studied by changing pH value of protein solution. At the last step, the mechanism deduced from lysozyme was studied
whether it is available or not for other proteins lacking tryptophan residue: ribonuclease A.

**I-5 References**


[I-2] McRee, D. Practical Protein Crystalography, **1999**.


Growth 1999, 200, 575.


Target identification and validation

Structure determination

Structure-based drug design

Leading compounds

Optimized leading candidates for trial

Scheme I-1 Flowchart of structure-based drug design
<table>
<thead>
<tr>
<th>Structure</th>
<th>Drug name</th>
<th>Mechanism</th>
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</thead>
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</tr>
<tr>
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<td></td>
</tr>
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</tr>
<tr>
<td><img src="image" alt="Structure" /></td>
<td>Tamiflu</td>
<td></td>
</tr>
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Table 1 Drugs derived from structure-based approaches
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<th>PUBS</th>
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Table I-2 The way to structure determination and number of proteins listed in Protein Data Bank (PDB)
Scheme I-2 Structure solution procedure by X-ray crystallography, showing steps as cloning, expression, solubilization, purification, crystallization and diffraction.
Figure I-1 The proportion of success on each experimental stage. Statistical results of structure solution projects; BSCG (circle), MCSG (triangle) and SECSG (square).
Figure I-2 Solubility curve of protein vs. salt concentration
Figure I-3 The free energy change ($\Delta G$) as a function of cluster radius ($r$).
Figure I-4 Size of critical cluster ($r^*$) dependence on supersaturation ($\beta$).
Figure I-5 Crystal growth process from cluster forming to crystal
Chapter II

Photochemically induced nucleation of Hen egg white lysozyme
II Photochemically induced nucleation of Hen egg white lysozyme

II-1 Introduction

II-1-1 Hen egg white lysozyme

Hen egg white lysozyme is a 14.4 kDa lytic enzyme. Figure II-1 shows three dimensional structure of Hen egg white lysozyme (PDB ID: 193L). Its structure was described by David Chilton Phillips in 1965 when he got the first 2 Å resolution image [II-1]. Figure II-2 shows the molecular sequence of lysozyme with consisting of 129 amino residues and 4 disulfide bridges. The enzymatic function of lysozyme is illustrated in Scheme II-1 [II-2]. The function is attacking bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. The amino acid side chains glutamic acid 35 (Glu35) and aspartate 52 (Asp52) have been found to be critical to the activity of this enzyme. Glu35 acts as a proton donor to the glycosidic bond, cleaving the C-O bond in the substrate, whilst Asp52 acts as a nucleophile to generate a glycosyl enzyme intermediate. The glycosyl enzyme intermediate then reacts with a water molecule, to give the product of hydrolysis and leaving the enzyme unchanged.

Lysozyme is widely used as a model system of protein for crystal graphic and photochemical studies because it is easily available.

II-1-2 Previous works of light induced nucleation of lysozyme

M. Terao and T. Okutsu reported that hen egg white lysozyme crystallization was enhanced by light irradiation [II-3]. In our laboratory, T. Okutsu et al. have investigated the photochemically induced nucleation of protein [II-4 - 7]. In past study, lysozyme solution was prepared for crystallization; 0.7 M NaCl containing 30 mg ml⁻¹ lysozyme in 0.1 M sodium acetate buffer solution at pH 4.5. Figure II-3 shows light irradiation set up for hanging drop
crystallization. The white light from 150 W Xe lamp irradiated to 10 μl droplet on the slide grass for 0 to 60 s. The light beam was parallelized go through a lens then vertically reflected to droplet by using prism. Figure II-4 shows the crystallization frequencies in one droplet changing irradiation time. They fitted the experimental crystal distribution with a Poisson law (Figure II-5). Up to 30 s of irradiation time, the experimental crystal distribution was well-fitted by a Poisson law, and they noted a significant discrepancy for an irradiation time of 60 s. This discrepancy was observed as saturation in the variation of the mean number of crystals in one droplet as a function of irradiation time, shown in Figure II-5. They concluded that nucleation frequencies are increased with increasing in irradiation time up to 30 s.

In parallel, to test wavelength dependence in this phenomenon, they selected different wavelengths of the Xe lamp emission (280, 300, and 400 nm) with a monochromator. The bandwidth of the 280, 300, and 400 nm light is ±5 nm. These wavelengths correspond to maximum (280 nm), edge (300 nm), and no (400 nm) absorption for lysozyme (Figure II-6 (a)). For these experiments, the irradiation time was increased up to 210 s because the light intensity decreases due to wavelength selection by a monochromator. Results are presented in Figure II-6 (b); the numbers of crystals are highly correlated with the absorption spectrum of lysozyme: no effect of irradiation at 400 nm on the nucleation of lysozyme and an increase of the nucleation frequency for irradiation at 280 and 300 nm, respectively. A maximum is observed at 280 nm. Because the absorption spectrum produces electronic transition of lysozyme, they conclude that light-induced nucleation was a photochemical process associated with electronic transitions.

Photoirradiation is known to cause denaturation of lysozyme. The activities were measured by the ML method. Figure II-7 shows photoirradiation time dependence of enzymatic activity of lysozyme. Irradiation times are 0, 30, 60, 120, and 180 s. The activity did not change until an irradiation time of 30 s and decreased linearly for irradiation times greater than 30 s. The
unchanged enzyme activity up to 30 s irradiation suggests the presence of a long-lived intermediate before lysozyme denaturation. This intermediate is assumed to play a significant role in nucleation and denaturation of lysozyme and is probably able to go back to lysozyme or to produce denaturated lysozyme by absorbing another photon.

To confirm the presence of this intermediate, the enzyme activity was measured for different irradiation light intensities. Figure II-8 (a) shows the evolution of the lysozyme activity obtained by the ML method for 120 s irradiation as a function of light intensity. The activity did not change linearly with irradiation intensity but is well-fitted by a parabolic law as shown in Figure II-8 (a). Figure II-8 (b) shows the logarithmic plot of the deactivation against light intensity. The slope of the logarithmic plots is 1.4 ± 0.6, which indicates that the deactivation took place by a one-photon process and by a two-photon process. They, here, pay attention to the two-photon deactivation process. Two types of photochemical two-photon processes are known; one occurs simultaneously with two-photon absorption and the other with stepwise photon absorption. Simultaneous two-photon absorption can be ruled out because the photon density of the Xe lamp is typically 108 times smaller than a pulsed laser, which induces simultaneous two-photon absorption. Stepwise two-photon deactivation suggests that denaturation occurs through an intermediate produced by one-photon absorption. From these result they concluded that lysozyme is stepwise denatured by photoirradiation, and expected that the mechanism of light induced the nucleation of lysozyme relate the photochemical intermediate. To summarize, they propose the one-photon-induced nucleation mechanism in Scheme II-2 [II-3, 4].

In this Chapter II, the mechanism of photochemically induced nucleation of lysozyme was investigated. To clarify the photochemical properties of lysozyme, electronic spectra measurements were carried out. To observe the progress of photochemical reaction by UV light irradiation, the generation of white turbidity was carried out on the slide glass. To avoid
non-photochemical effect on the crystallization by use of light irradiation, novel crystallization technique liquid-seeding was developed. To identify the photochemical intermediate of lysozyme in Scheme II-2, transient absorption experiments were carried out. To consider the mechanism of photochemically induced nucleation, enzymatic activity measurements with scavenging the intermediate using visible light irradiation and crystallization experiments with using scavenging technique were carried out. To detect the dimer as a photochemical product, SDS-PAGE was carried out.
II-2 Experimental section

II-2-1 Sample preparation and experimental apparatus

Hen egg white lysozyme was purchased from Seikagaku (6 times recrystallized lot E02Z04) and was used without further purification. Sodium acetate trihydrate, acetic acid, sodium chloride, L-tryptophan, L-tyrosine and L-phenylalanine, all of them GR-grade, were purchased from Wako pure chemical. Sodium acetate trihydrate and acetic acid were dissolved in ultra-pure water (Milli pore, Milli-Q) and used as a buffer solution: NaAc buffer, 50 mM pH 4.3. Prepared buffer solution was adjusted pH by digital pH meter (model 15, Fisher Scientific). Buffer solutions were used for sample preparation and carried out at room temperature. The concentration of lysozyme solution was determined using an extinction coefficient of 2.64 ml mg$^{-1}$ cm$^{-1}$ at 280 nm [II-8]. Absorption and emission spectra were recorded on a spectrometer (Hitachi U-3300) and a fluorescence spectrometer (Hitachi F-4500).

II-2-2 Light irradiation

The light source used for the preparation of irradiation samples and in the photochemically induced nucleation experiments, was a xenon short arc lamp (USHIO UXL-300D, 300 W). Figure II-9 shows UV light irradiation apparatus. The light beam from the lamp was passed through water (light pass length = 20 mm), to cut the near-infrared radiation, and a quartz lens ($f$=100 mm, 50 mm diameter), which focused to the entrance slit of monochrometor (JASCO, CT-10). Figure II-10 shows radiation spectra from a 300 W Xe lamp (dotted line) through a monochromator at 280 nm (broken line). The light beam was extracted at 280 nm, which was used to directly irradiate sample solution in the cell. Figure II-11 shows the apparatus for two-color light irradiation experiments. The light beam from a 500 W Xe lamp (USHIO UXL-500D, 500 W) was cut off under 350 nm UV radiation using a cut off filter and visible
light (Vis) beam was extracted. The Vis irradiation apparatus was set up co-axial at anti position to UV irradiation apparatus.

II-2-3 Crystallization Experiment

Crystallization Experiments were carried out in incubation room at 20 °C. The microbatch crystallization experiments were carried out in a 72-wells microbatch plate, purchased from Hampton Research. The microbatch plate was covered with paraffin oil before adding the droplets. Figure II-12 shows liquid-seeding technique. As a seed solution, sodium chloride in buffer solution was mixed with lysozyme then irradiated. The seed solution was dropped on the plate then lysozyme solution was dropped and mixed by micropipette at 20 °C. To avoid vapor diffusion from sample solution through parafin oil to out of plate, the plate was sealed by silicone glees. The prepared microbatch plate was stored at 22 °C.

II-2-4 Transient absorption measurements

For transient absorption measurements of lysozyme and its aromatic amino residues, a Nd3+:YAG laser (Lotis, 30 ns fwhm, 3 mJ pulse-1, 10 Hz) was used as an excitation light source at 266 nm. Figure II-13 shows transient absorption spectra measurement setup. The samples were flowed through a quartz cell with a flow rate of ca. 40 mL min⁻¹. The produced intermediates by the excitation laser absorb the white light through a water (light pass length = 3 mm) from a 500 W xenon short ark lamp. 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.
II-2-5 Enzymatic activity measurements by ML methods

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. The experimental procedure was described in the literature [II-9]. *Micrococcus lysodeikticus* (lot 052K8618) was purchased from Sigma industry. Lytic activity was performed with suitable aliquots of the diluted lysozyme solution (0.1 mL completed to 10 mL of water) in the reaction mixture (0.25 mg of dried *Micrococcus lysodeikticus* cells per milliliter of 40 mM sodium phosphate buffer, pH 7.0). I recorded the decrease in optical density of the turbid cell suspension at 450 nm with a spectrophotometer (Hitachi U-3300). Mean values of the activities at different concentrations of lysozyme were determined. The assays were linear over 5 min at room temperature. The specific activity of the lysozyme solution was determined to be 51 000 U g$^{-1}$.

II-2-6 SDS-PAGE experiment

The SDS-PAGE experiment was carried out using a slab minigel electrophoresis unit (Nihon Eido, NA-1020, CN-1010) with 15% resolving and 0.05% concentrating gels. Tris-Gly buffer solution (containing 0.4% SDS) was used as the electrode solution. Five μl of the sample solution were loaded onto each lane of gel. The gel was impressed on power current at 8 mA for 3 h. The gel was stained silver stain kit from Wako pure chemical.
II-3 Results and Discussion

II-3-1 Steady state electronic spectra measurements

To clarify the photochemical properties of lysozyme, the electronic spectra of lysozyme, L-tryptophan (Trp), L-tyrosine (Tyr), L-phenylalanine (Phe) were measured. Figure II-14 shows the absorption and emission spectra of lysozyme (a), Trp (b), Tyr (c) and Phe (d). The absorption and emission spectra are indicated by dashed and solid line, respectively. Trp, Tyr and Phe are aromatic amino acids that are expected to participate in the photochemical reactions of lysozyme. Lysozyme and these amino acids in Figure II-4 exhibit fluorescence which wavelengths indicate the emission peak at (a) 340, (b) 350, (c) 310 and (d) 280 nm. The lysozyme (a) emission spectrum was identical to the region of (b) Trp emission spectra and differed from the (c) Tyr and (d) Phe emissions. These results suggest that the emission of lysozyme is ascribable to excited state of Trp. Lysozyme emission is known to consist of the emission from excited Trp, which is partly generated through intermolecular energy transfer from Try, Phe and Trp to 62nd Trp residue [II-10 - 12]. The lysozyme molecule contains six Trp, three Tyr and three Phe residues as aromatic residues (Figure II-1 and II-2). Since the Trp appears at a longer wavelength than that of Tyr and Phe, the excited state energy of Trp showed lower than those of Tyr and Phe. If the Tyr or Phe residual absorbs a photon, the excited state energy can be transferred to 62nd Trp residue through intramolecular Förster-type energy transfer.

II-3-2 Slide grass test

To observe the progress of photochemical reaction by UV light irradiation, the generation of white turbidity was carried out on the slide glass. 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 II-15 (a)-(c) shows photographs of the lysozyme solutions in this setup. NaCl concentrations were
indicated below the photographs. The lysozyme concentrations were adjusted at 30 mg mL\(^{-1}\). In the lysozyme solution without NaCl, no apparent change was observed in Figure II-15 (a), whereas in the solutions containing NaCl in Figure II-15 (b) and (c), white turbidity appeared in the irradiated part. It is 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. When the NaCl concentration was increased, the white turbidity was 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.

II-3-3 Development of crystallization technique

With going forward confirmation of the mechanism of crystallization enhancement by photochemical reaction, it is necessary to remove affects of non-photochemical reactions. Therefore novel crystallization technique was developed.

Here, two type crystallization methods in popular use are presented [I-1 - 3]. Figure II-16 shows hanging drop vapor diffusion crystallization set up (a) and its time profile of concentration in droplets (b). Figure II-17 shows oil batch crystallization set up (a) and its time profile of concentration in droplets (b). These method differ time profile of concentration in droplet. In the vapor diffusion method, both concentration of protein and precipitant in droplet is increased with doing vapor-liquid equilibrium between droplet and reservoir solution because reservoir solution has higher concentration of precipitant than droplet. At last, protein concentration in droplet is decreased to equilibrium when droplet solution reaches the nucleation region. In the batch crystallization method, only protein concentration is decreased
when nucleation occurred.

Previous crystallization technique for the photochemically induced nucleation of lysozyme had some problems. The hanging drop vapor diffusion method was used for the crystallization and directly irradiated to droplet on the circle slide grass [II-3]. In this case, spontaneous vapor diffusion from the droplet to atmosphere occurred during light irradiation and photon absorb only surface of droplet because the concentration of lysozyme is too high to directly irradiate. Unwished nucleation was occurred due to increase supersaturation by the spontaneous vapor diffusion. The unwished nucleation and photon-absorption on the surface of droplet complicated problem.

The developed the novel crystallization technique is used as seeding which separated nucleation from crystal growth process and we named it “liquid-seeding”. Figure II-12 shows liquid-seeding technique for photochemically induced nucleation of protein. The liquid-seeding technique separates two steps; nucleation and growth step were separated from the crystallization. Figure II-18 shows solution condition for photochemically induced nucleation of lysozyme using liquid-seeding technique, showing a (a) nucleation step and (b) growth step. The solid line in Figure II-18 shows solubility curve. In the nucleation step, lysozyme solution was prepared at metastable condition in sample cavity cell. UV light from Xe lamp is irradiated to the metastable solution which does not cause spontaneous nucleation. In the growth step, irradiated sample and pure lysozyme solutions are mixed on the batch plate covered with inert paraffin oil. To avoid the unwished nucleation by down in temperature, these experimental steps of liquid-seeding are carried out at 20 °C then accomplished plate is stored at 22 ºC. I also checked the temperature rise of the solution during the irradiation. Figure II-19 shows the temperature change of the solution in the 1 cm × 1 cm × 4 cm dimension optical cell during the irradiation. Temperature raised from 22.22 to 24.75 °C during 180 s of irradiation. The temperature rise can be estimated at 3 °C at most.
during the irradiation, indicating that the heat of the solution is not responsible for the nucleation because up in the temperature decrease the supersaturation in seed solution.

Figure II-20 shows photographs of the droplets of irradiated solutions and pure lysozyme solution observed after 24 h. Irradiation time was 0 (a), 60 (b) and 180 s (c). In the droplets without irradiation, no crystal was observed in all the droplets. On the other hand, lysozyme crystals appeared in the droplet of irradiated solution. The number of crystal was increased with increasing in irradiation time. From these results, photochemically induced nucleation was confirmed.

For estimation of crystal quality using liquid-seeding technique, diffraction studies on the crystals were carried out at BL44B2, Spring-8 with ADSC Q210 as detector. In the crystallographic experiments, I used the samples without and with 60 s irradiation in nucleation step. Figure II-21 shows diffraction pattern of crystals of (a) without and (b) UV irradiated samples. The square in Figures was expanded below. The highest diffractions were up to (a) 1.55 and (b) 1.50 Å resolution. The space group of both crystals belonged to tetragonal \( P4_{3}2_{1}2 \), with unit-cell dimensions of \( a = b = 78.86 \) or \( a = b = 78.81 \) and \( c = 37.01 \) Å. These data are collected in Table II-1 and loss of crystal quality by damage of UV light irradiation is not observed.

These results proved that liquid-seeding technique for nucleation of lysozyme induces non-photochemical but photochemical affect and the crystal quality is not charge to common microbatch crystallization methods.

**II-3-4 Transient absorption measurements**

In order to identify the photochemical intermediate in Scheme II-2, transient absorption experiments were carried out. To avoid denaturation of lysozyme by a two-photon absorption mechanism, sample solutions were flowed rapidly (40 mL min\(^{-1}\)) through a quartz small
cavity cell. Figure II-22 shows transient absorption spectra of (a) lysozyme, (b) Trp, (c) Tyr and (d) Phe in 50 mM NaAc buffer solution at room temperature. Spectra were recorded at 2 (dashed line) and 64 μs (solid line) after laser flash. The concentrations of sample solutions were adjusted to the optical density of 1 by using a flow-cell of 4 mm optical pass length. The triplet state was not detected because the sample solutions were saturated with O2 gas [II-13]. These spectra are compared, the transient species of (a) lysozyme having peak at 520 nm are good similar with (b) Trp and differ from (c) Tyr having typically phenoxy radical peak around 400 nm [II-14] or (d) Phe having no peak in this wavelength region. These results were in good agreement with reports [II-14 - 21]. The spectra of (a) lysozyme are composed of two different species; one is a fast-decaying species having absorption in the range 300-600 nm the lifetime of which was shorter than 4 μs. The fast-decaying species is assigned as disulfide (-S-S-) electron adducts from the literature [II-23]. The other is a long-lived species having absorption peaks at 520 nm. Transient species having absorption peak at 520 nm of Trp corresponded to neutral tryptophanyl radical (Trp●) existing radical center on the nitrogen atom of indole-ring [II-17].

The generation of residual Trp● in lysozyme by photochemical reaction illustrated in Scheme II-3. The initial photochemical reaction of tryptophan residue of lysozyme is photo-ionization leading to radical cation (Trp●+) and hydrated electron. The Trp● of the residual of lysozyme formed from Trp●+ by deprotonation [II-19, 24]. The ejected electron is immediately hydrated, which is called hydrated electron showing absorption band over 650 nm [II-15]. Trp●+ has absorption peak at 570 nm.

The decay time profile at 520 nm is shown in Figure II-23. Half-lifetime of the intermediate of lysozyme and Trp were estimated to be 1 ms and 100 μs. The reciprocal plot of the time profile showed a linear dependence, the intermediate species decays through a second-order process. The observed half-lifetime of lysozyme (τ 1/2 = 1 ms) shows that ca. 250 collisions
occur within the intermediate half-lifetime [II-25]. It was reported that 10 lysozyme molecules are necessary to form the critical nucleus in the same condition [I-24]. And if one collision per 250 collisions can form dimer (radical-radical), rate determining step on the nucleation can over because the smallest cluster of 2 molecules was surface/volume disadvantage is the largest.

Thus intermediate of lysozyme in Scheme II-2 having absorption peak at 520 nm confirms residual tryptophanyl radical (Trp\(^\cdot\)). Transient absorption results suggests the following mechanism; the produced residual Trp\(^\cdot\) by UV light irradiation produced covalently bonded dimer, which forms cluster and finally grows to crystal (SchemeII-4).

**II-3-5 Enzymatic activity measurements**

To confirm the mechanism in Scheme II-4, I performed with quenching Trp\(^\cdot\) by visible (Vis) light irradiation from Xe lamp. It is expected that UV light produces residual Trp\(^\cdot\) which is able to be scavenged by Vis light. Figure II-24 shows the absorption spectra of lysozyme (solid line) and residual Trp\(^\cdot\) (dashed line), showing regions of UV (a) and Vis radiation (b) from Xe lamp. These spectra show to be able to selectively irradiate to the species.

Before carrying our crystallization experiments, I confirmed to be able to quench Trp\(^\cdot\) by Vis light irradiation from 500 W Xe lamp. For quenching experiments by UV and Vis light irradiation, enzyme activity measurements by ML method as a function of irradiation time with two colors of excitation were carried out. Figure II-25 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 [II-3], 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 suggests the two-step deactivation denaturation mechanism in Scheme II-2. From these results, it is confirmed that two-color light irradiation is controllable to existence of the residual Trp*.

II-3-6 Crystallization by two-color irradiation

To confirm whether the intermediate induces nucleation as suggested in Scheme II-2. It was simultaneously carried out that four nucleation experiments with or without irradiation by UV (280 nm) or visible (>350 nm) light or a combination of the two as described as liquid-seeding technique. Figure 30 shows photographs of droplets under paraffin oil. Figure II-26 (a) shows a droplet without irradiation. No crystal appears in the droplet. Figure II-26 (b) shows a droplet by UV (280 nm) light irradiation for 120 s. 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 photochemically induced nucleation has occurred. Figure II-26 (c) shows a droplet by visible (>350 nm) light irradiation. Lysozyme has no absorption in the visible (>350 nm) wavelength range; this result is the same as the result without irradiation. Finally, Figure II-26 (d) 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 photochemically induced nucleation mechanism proposed in Scheme II-2.

II-3-7 Dimer detection by SDS-PAGE experiment
I expect that the residual Trp\(\cdot\) produced by UV light irradiation causes dimerization, because general organic compound’s radicals are dimerized themselves. To detect the covalently bonded dimer of lysozyme, SDS-PAGE was carried out. In parallel consideration, amount of the dimer was estimated from results of transient absorption spectrum measurement.

Figure II-27 shows a photograph of gel. Lanes 1 to 4 are lysozyme solution containing NaCl (0.1 M), Lane 5 is lysozyme dimer, Lanes 6 to 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. 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 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 to 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.

To estimate amount of the produced dimer by photochemical reaction and relationship of liquid-seeding technique, amount of residual Trp\(\cdot\) in lysozyme was calculated from the radiation energy of Xe lamp and transient absorption spectrum of lysozyme. In Figure II-22 (a), optical density of residual Trp\(\cdot\) at 520 nm showed approximately 2.0\(\times\)10\(^{-3}\). It has been reported that the molecular extinction coefficient \(\epsilon\) of Trp\(\cdot\) at 520 nm is 1800 M\(^{-1}\) cm\(^{-1}\). From these values, 1.1 \(\mu\)M of residual Trp\(\cdot\) was produced by laser pulse irradiation at 3 mJ.
Assuming the produced residual Trp\(^\bullet\) is perfectly dimerized, 3 mJ laser pulse produces 0.55 \(\mu\)M of covalently bonded dimer. From radiation spectrum of Xe lamp through a monochromator (broken line) in Figure II-10, we calculated that radiation intensity in 250–300 nm wavelength region is approximately 150 \(\mu\)W. The UV light irradiation for 60 s with using monochromator provides photon energy of approximately 9 mJ then 1.65 \(\mu\)M of dimer was produced. The liquid-seeding technique was used equal volume of precipitant solution containing the dimer (1.4 M NaCl and 1 mg ml\(^{-1}\) lysozyme) and pure lysozyme (39 mg ml\(^{-1}\)). From these calculation results, we estimated that the dimer in a droplet with irradiation 60 s existed about 1.2% and crystal appeared in droplet. T. Matsui \textit{et al.} reported impurity effect of lysozyme. The addition of chemically modified F-lysozyme (approximately 0.17%) having fluorescent probe of functional group as an impurity drastically promoted nucleation [II-27].

It is concluded that the promoted nucleation of lysozyme was heterogeneous nucleation by addition of impurity. The result of SDS-PAGE suggests that covalently bonded dimer was produced from residual Trp\(^\bullet\) by UV light irradiation and the dimer induces nucleation of lysozyme.

**II-4 Summary**

In this Chapter, the mechanism of the photochemically induced nucleation of Hen egg white lysozyme was investigated. In lysozyme system, photochemically induced nucleation brought about the photochemical reaction of the Trp residual groups instead.

It was confirmed that the chromophore of lysozyme is tryptophan. The white turbidity of lysozyme was produced by UV light irradiation. This result suggested aggregation of lysozyme was enhanced by UV light irradiation.

To avoid the non photochemical affect on the experiment of the photochemically induced
nucleation of lysozyme, liquid-seeding was developed. In crystallization experiments by use of liquid-seeding technique, the number of crystals increased with increasing in irradiation time for 0, 60 and 180 s. The lattice parameters of crystal obtained by use of liquid-seeding technique dose not change to lattice parameter by use of common microbatch crystallization method.

The intermediate in Scheme II-2 is produced by one-photon absorption, and resulting residual tryptophanyl radical. The lytic activity of coupling UV to Vis light irradiated solution increased the efficiency of denaturation process than only UV light irradiated solution. The results of activity measurements suggested that the two-step denaturation mechanism relate residual tryptophanyl radical. From crystallization experiments, lysozyme crystallization was enhanced by only UV light irradiation but not enhanced by coupling UV to Vis light irradiation. The photograph of SDS-PAGE showed that covalently bonded dimer (Mw = 28 kDa) was produced by UV light irradiation.

It is summarized in Scheme II-4 that covalently bonded dimer was produced from residual tryptophanyl radical by photochemical reaction then it plays important role as the smallest cluster in early stage of nucleation process.
II-5 References


Figure II-1 Hen egg white lysozyme three dimensional structure
Figure II-2 Molecular sequence of lysozyme
Scheme II-1 Enzymatic reaction of lysozyme
Figure II-3 Irradiation setup for light induced nucleation, using previous work
Figure II-4 The crystallization frequencies in one droplet changing irradiation time.
Figure II-5 Mean number of crystals in one droplet function as irradiation time.
Figure II-6 Absorption spectrum of lysozyme (a) and photographs of hanging droplets observed after 48 h. Irradiation wavelengths are indicated below the photographs.
Figure II-7 Light irradiation action spectra of lysozyme. Enzymatic activity
Figure II-8 Enzymatic activity (semi log) and power dependence
Scheme II-2 Expected mechanism of photochemically induced nucleation of lysozyme
Figure II-9 UV light irradiation apparatus
Figure II-10 Radiation spectra from 300 W Xe lamp (dotted line) through a monochromator at 280 nm (broken line).
Figure II-11 Irradiation apparatus for two-color excitation experiments
Figure II-12 Liquid-seeding technique
Figure II-13 Experimental setup for transient absorption
Figure II-14 Steady state electronic spectra of lysozyme and aromatic amino acids
Figure II-15 Photographs of the lysozyme solution. NaCl concentrations in solution indicate below the photographs.
Figure II-16 Hanging drop vapor diffusion crystallization set up (a) and its time profile of concentration in droplets (b)
Figure II-17 Oil batch crystallization set up (a) and its time profile of concentration in droplets (b)
Figure II-18 Crystallization condition for photochemically induced nucleation of lysozyme using liquid-seeding technique. Concentration of sample solution for nucleation (a) and crystal growth (b)
Figure II-19 Temperature changing of sample solution by light irradiation
Figure II-20 Photographs of the droplets irradiated solution and pure lysozyme solution observed at 24 h after. Irradiation time was 0 (a), 60 (b) and 180 s (c).
Figure II-21 Diffraction pattern of crystals of without (a) and UV irradiated samples (b).
<table>
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<th>UV</th>
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<td>P4\textsubscript{3}2\textsubscript{1}2</td>
</tr>
<tr>
<td>Cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b (Å)</td>
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<td>78.86</td>
</tr>
<tr>
<td>c (Å)</td>
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<td>37.01</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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<td>1.50</td>
</tr>
<tr>
<td>Rmerge (%)</td>
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<td>8.4 (27.6)</td>
</tr>
<tr>
<td>I/\sigma(I)</td>
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<td>23.0 (9.5)</td>
</tr>
<tr>
<td>Redundancy</td>
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<td>13.5 (13.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100.0 (100.0)</td>
<td>100.0 (100.0)</td>
</tr>
</tbody>
</table>

Table II-1 Diffraction date of crystals with using liquid-seeding technique
Figure II-22 Transient absorption spectra of lysozyme (a), Trp (b), Tyr (c) and Phe (d).
Scheme II-3 Reaction of Residual tryptophanyl radical

\[ \lambda_{\text{max}} = 520 \text{ nm} \]

\[ \tau_{1/2} = 1 \text{ ms} \]
Figure II-23 Decay time profile of intermediate of lysozyme (a) and tryptophan (b) at 520 nm. Half-lifetime ($\tau_{1/2}$) of lysozyme and tryptophan indicate approximately 1 ms and 100 μs, respectively.
Scheme II-4 photochemically induced nucleation of lysozyme
Figure II-24 the absorption spectra of lysozyme (solid line) and residual Trp$^\circ$ (dashed line), showing regions of UV (a) and Vis radiation (b) from Xe lamp.
Figure II-25 Enzymatic activity dependence of lysozyme function as irradiation time (a.u. = arbitrary unit): irradiated with Vis (open circle), UV (closed square) and UV and Vis simultaneously. Error bars are estimated ±3% for each experimental date.
Figure II-26 Photographs of droplets of lysozyme, showing without irradiation (a), with UV irradiation for 120 s (b), with Vis irradiation for 120 s (c) and with simultaneous irradiation of UV and Vis for 120 s
Figure II-27 Photograph of SDS-PAGE gel of Lysozyme.
Chapter III

pH dependence of photochemically induced nucleation of lysozyme
III pH dependence of photochemically induced nucleation of lysozyme

III-1 Introduction

In Chapter II, the mechanism of the photochemically induced nucleation of lysozyme was investigated. 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 [II-3]. Neutral radicals of tryptophan residue of lysozyme (RTrp●) were observed in buffer solution at pH 4.3 by transient absorption measurements [II-3, 4]. Photochemical dimerization of lysozyme was also observed by SDS-PAGE for this solution [II-5]. Based on these results, it was suggested that the dimer plays a role of the smallest cluster in the crystallization. Scheme III-1 shows the mechanism of PIN of lysozyme. Grossweiner et al. reported that the initial photochemical reaction of tryptophan residue of lysozyme is photo-ionization leading to the generation of radical cation (RTrp●+) and hydrated electron [II-15]. The RTrp●+ releases a proton to give the RTrp● [III-1]. The RTrp●+ is considered to be in equilibrium with RTrp●, since radical cations of L-tryptophan was found to be in equilibrium with the neutral radical. Therefore, quantity of RTrp● should depend on the pH value of the lysozyme solution; if the pH value of the solution is higher than the pKa value of RTrp●+, quantity of RTrp● is large and the dimer formation may become efficient to enhance the nucleation. The pKa value of the radical cation of L-tryptophan was reported to be ca. 4.3, but the pKa value of RTrp●+ has not been reported yet [II-21]. We, here, demonstrate the results of PIN experiments of lysozyme solution at several pH values. The pKa value of RTrp●+ 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 pKa value.

Thus, there remains a problem to understand how the formation of the dimer does enhance the nucleation. One of the way to solve this problem is to know the effects of covalently
bonded dimer by varying its concentration. It is, however, not possible to control the concentration of the dimer by changing the irradiation time because light irradiation produced not only dimer but also denatured-species, the latter disturbing crystallization of protein.

III-2 Experimental section
Hen egg white lysozyme was purchased from Seikagaku (6 times recrystallized lot E02Z04) and was used without further purification. L-tryptophan (TrpH), sodium chloride (NaCl), hydrochloric acids (HCl), sodium hydrate (NaOH), potassium dihydrogen phosphate (KH$_2$PO$_4$), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), citric acid monohydrate (Citric), sodium tetraboronic acid decahydrate (Na$_2$B$_4$O$_7$) and potassium chloride (KCl), all reagents (GR-grade), purchased from Wako Pure Chemical Ind. Ltd. The UV-Vis wide range buffer (WR buffer) used as a solvent; KH$_2$PO$_4$, Tris, Citric, Na$_2$B$_4$O$_7$ and KCl were dissolved into ultra-pure water (Milli pore, Milli-Q) and pH was adjusted by adding HCl or NaOH aqueous solution. All solvent concentrations were prepared at 100 mM [III-2].

III-3 Results and Discussion

III-3-1 Transient absorption measurements
To estimate the pKa value of RT$_{\text{pH}}^{\text{H}^+}$, transient absorption spectra of lysozyme and L-tryptophan (TrpH) were measured by nanosecond laser flash photolysis.

First, pKa of the radical cation of tryptophan (TrpH) was estimated. Figure III-1 shows pH dependence of transient absorption spectra of TrpH in WR buffer solution. The spectra were recorded at 64 μs after the laser flash. The acidity of solution was adjusted to pH 2.0 (a), 3.0 (b), 4.3 (c) and 5.0 (d), respectively. Figure III-1 (a) and (b) show an absorption band in the region of 500 - 650 nm. Figure III-1 (d) shows an absorption band in the region of 450 - 600 nm. While, Figure III-1 (c) shows an absorption band in the region between those of (a) and
All these spectra show a broad absorption band due to hydrated electron in the wavelength region longer than 650 nm. Bent and Hayon reported that the absorption of Trp$^{**}$ shows a peak at ca. 570 nm and Trp* shows at ca. 520 nm. They also reported that pKa value of Trp$^{**}$ was approximately 3.0 at room temperature. In this study, Trp$^{**}$ was observed in the spectra of pH 2.0 (Figure III-1 (a)) and pH 3.0 (Figure III-1 (b)), and Trp* was at pH 5.0 (Figure III-1 (d)). The spectrum at pH 4.3 is considered to be due to the mixture of Trp$^{**}$ and Trp*. These results suggest that the pKa value of Trp$^{**}$ is ca. 4.3, being in agreement with the reported value [II-21].

Next, transient absorption spectra of lysozyme were studied. Figure III-2 shows pH dependence of the transient absorption spectra of lysozyme in WR buffer solution. The spectra were recorded at 64 μs after the laser flash. The acidity of solution was adjusted to pH 2.0 (a), 3.0 (b), 4.3 (c) and 5.0 (d), respectively. Figure III-2 (a) shows an absorption band in the region of 500 - 650 nm. Figure III-2 (c) and (d) show an absorption band in the region of 450 - 600 nm. The main intermediates of lysozyme observed are RTrp$^\text{\ Brotherhood}$ ($\lambda_{\text{max}} = 570$ nm), RTrp$^\text{\ Brotherhood}$ ($\lambda_{\text{max}} = 520$ nm) and hydrated electron ($\lambda_{\text{max}} > 650$ nm) [II-15]. While, in Figure 1 (b) 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 L-tryptophan upon the photolysis shown in Figure III-2.7-10 The main absorption species at pH 2.0 and 4.3-5.0 are ascribed to RTrp$^\text{\ Brotherhood}$ and RTrp$^\text{\ Brotherhood}$, respectively. As a result, the pKa value of RTrp$^\text{\ Brotherhood}$ of lysozyme is estimated to be ca. 3.2. Thus, the pKa value of RTrp$^\text{\ Brotherhood}$ is confirmed to be a little small compared with that of the radical cation of L-tryptophan.

III-3-2 Dimer detection by SDS-PAGE

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 III-3. 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.

III-3-3 pH dependence of photochemically induced nucleation of lysozyme

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 III-4 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 Figure III-4 (a) and (b), 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 [III-3], 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, 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 pKa value of RTrp$^\ddagger+$, the quantity of RTrp$^\ddagger$ at pH 5.0 is estimated to be approximately 5 times greater than that at pH 4.3. If all of the RTrp$^\ddagger$ 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.

III-4 Summary

In this Chapter, the mechanism of photochemically induced nucleation of lysozyme was investigated by making use of pH dependence of solution. The pKa value of residual Trp$^{**}$ in lysozyme was estimated to be 3.2 by transient absorption measurements and SDS-PAGE experiment. 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 pKa value of RTrp$^\ddagger+$, the quantity of RTrp$^\ddagger$ at pH 5.0 is estimated to be approximately 5 times greater than that at pH 4.3. If all of the RTrp$^\ddagger$ 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$^\ddagger$ is generated in the solution with pH value higher than pKa value of RTrp$^\ddagger+$.

III-5 References

Scheme III-1 The mechanism of photochemically induced nucleation of lysozyme
Figure III-1 pH dependence of transient absorption spectra of TrpH in WR buffer solution. The spectra were recorded at 64 ms after the laser flash. The acidity of solution was adjusted to pH 2.0 (a), 3.0 (b), 4.3 (c) and 5.0 (d), respectively.
Figure III-2 pH dependence of transient absorption spectra of Lysozyme in WR buffer solution. The spectra were recoded at 64 ms after the laser flash. The acidity of solution was adjusted to pH 2.0 (a), 3.0 (b), 4.3 (c) and 5.0 (d), respectively.
Figure III-3 Photograph of SDS-PAGE gel of lysozyme changing pH. Sample solutions were irradiated for 30 min or nothing.
Figure III-4 Photographs of PIN of lysozyme changing pH and irradiation time.
Chapter IV

Photochemically induced nucleation of Bovine pancreatic ribonuclease A
IV Photochemically induced nucleation of Bovine pancreatic Ribonuclease A

IV-1 Introduction

The photochemically induced nucleation technique suggests the possibility of breakthrough the bottleneck in protein X-ray crystallography for structure-based drug design. In Chapter III, it was summarized that covalently bonded dimer was produced from neutral radical of tryptophan residue of lysozyme by photochemical reaction then it plays important role of the smallest cluster in early stage of nucleation process. However, I don’t know what Trp residue lacked protein induces nucleation by photochemical reaction. The study of Trp lacked protein is the most important to clarify the mechanism of photochemically induced nucleation whether phenomenon affecting protein in general. For this purpose, bovine pancreatic ribonuclease A (RNaseA) was selected.

RNaseA is an enzyme capable of cleaving the phosphodiester bond between the nucleotide subunits of nucleic acid. Crystallization procedure of RNaseA has been already reported [IV-1 - 4]. Figure IV-1 shows three dimensional of RNaseA (PDB ID: 1FS3), structure determination by X-ray crystallography. Figure IV-2 shows molecular sequence of RNaseA. RNaseA consists of 124 amino acid residues which contain six Tyr and three Phe as chromophores and molecular weight is approximately 13,800 Da. The photochemical process of RNaseA has been reported [II-18]. The enzymatic reaction and its relating residues of RNaseA are shown in scheme IV-1 [IV-5].

In this chapter, I investigated the photochemical properties of RNaseA by means of steady-state and transient spectroscopy from the viewpoint of photochemically induced crystallization. An electrophoresis experiment for observing the photochemical dimmers was also carried out to examine the crystallization mechanism.
IV-2 Experimental section

IV-2-1 Sample preparation

Genomic-research-grade RNaseA was purchased from Wako pure chemical and used without further purification. Sodium acetate, acetic acid, tyrosine (Tyr), phenylalanine (Phe), sodium chloride (NaCl) and ammonium sulfate (A.S.), all of them GR-grade, were purchased from Wako Pure Chemicals. Sodium acetate and acetic acid were dissolved in ultra-pure water (Milli pore, Milli-Q) and used as the buffer solution (NaAc buffer, 50 mM, pH 5.5). The prepared solution was centrifuged and filtered through a 0.45-μm membrane filter (NALGENE) before the crystallization experiment. The RNaseA concentration was determined using an extinction coefficient of 0.70 L g⁻¹ cm⁻¹ at 280 nm [IV-6]. Sample preparation was carried out at room temperature.

IV-2-2 Crystallization experiment and UV irradiation apparatus

The batch crystallization experiment was carried out in a 72-well micro batch plate purchased from Hampton Research. The micro batch plate was covered with paraffin oil before adding the droplets. The droplets were irradiated for 0~300 s by UV light through the paraffin oil. The irradiated plate was sealed with silicone grease and stored at 20 ºC for one week.

The light source used for the preparation of the irradiation samples and in the photochemically induced crystallization experiment was a Xe lamp (USHIO, UXL 300D, 300 W). Figure IV-3 shows the light irradiation apparatus for the photochemically induced nucleation experiment. Figure IV-4 shows transmission spectra of band pass filter UVTF-33U. The light beam from the lamp is passed through water (light pass length = 20 mm), to cut the near-infrared radiation, and through a band pass filter (SIGMA KOKI, UVTF-33U), to cut the visible radiation. Figure IV-5 shows the radiation spectrum of 300 W Xe lamp (dashed line).
through a 33U band pass filter (solid line) and through a monochromator at 280 nm (broken line).

IV-3 Results and Discussion

IV-3-1 Steady state electron spectra measurements

The steady-state electronic spectra were measured. Figure IV-6 shows the absorption and emission spectra of RNaseA (a), Tyr (b) and Phe (c). The absorption and emission spectra are indicated by the dashed and solid line, respectively. Tyr and Phe are aromatic amino acids that are expected to participate in the photochemical protein reaction. Both of these amino acids emit fluorescence. The RNaseA (a) emission spectrum is identical to the Tyr emission spectrum (b) and differs from the Phe emission (c). These results suggest that the excited state of RNaseA is actually the excited state of Tyr. Lysozyme emission is known to consist of the emission from excited Trp residual groups, which is partly generated through intramolecular energy transfer from Phe or Tyr residuals [II-10]. In an RNaseA molecule, six Tyr and three Phe residues are contained as aromatic residues. As the emission from Tyr has a longer wavelength than that from Phe, the excited state energy of Tyr is lower than that of Phe. If the Phe residual absorbs a photon, the excited state energy can be transferred to Tyr through intramolecular Förster-type energy transfer.

IV-3-2 Transient absorption spectra measurements

To observe the photochemical intermediates of RNaseA, transient absorption experiments were carried out and the transient absorptions of Tyr and Phe were compared. Figure IV-7 shows the transient absorption spectra of RNaseA (a), Tyr (b) and Phe (c) in 50 mM sodium acetate buffer at pH 5.5. The spectra were measured 64 μs after the laser flash. The triplet state was not detected because the sample solutions were saturated with O₂ gas. Figure IV-7
(a) shows the RNaseA transient absorption spectrum with an absorption band shorter than 440 nm having vibrational bands of 410 and 390 nm. Figure IV-7 (b) shows the Tyr transient absorption spectrum, which is almost identical to the transient absorption spectrum of RNaseA and is the spectrum typically caused by phenoxy radicals. Figure IV-7 (c) shows the Phe transient spectrum with an absorption band shorter than 370 nm, which differs from the absorption bands of RNaseA (a) and Tyr (b). Scheme IV-2 shows photochemical reaction of tyrosyl neutral radical (TyrO●). A tyrosine (TyrOH) absorbs photon energy then excited state of tyrosine (TyrOH*) is produced. TyrOH* does electron ejection and deprotonation. As a result, TyrO● was produced and dimerize to di-tyrosine. Figure IV-8 shows conformation of covalently bonded di-tyrosine [IV-7, 8]. H. R. Shen et al. reported that there are three conformations of tyrosine and its distribution. The residual TyrO● in RNaseA also forms conformation is expected.

These results indicate that the photochemical intermediate of RNaseA is the residual Tyr radical in which the phenol group is converted into a phenoxy radical, and this finding agrees with the reports [II-14, 18]. It is expected that residual TyrO● of RNaseA forms covalently bonded dimer in a uniform manner of di-tyrosine.

**IV-3-3 Dimer detection by SDS-PAGE experiment**

Covalently bonded dimers are considered to behave as the smallest stable clusters in the early stage of the nucleation process. To confirm the presence of photochemically produced covalently bonded RNaseA dimers, an SDS-PAGE experiment was carried out. Figure IV-9 shows a photograph of the gel. Lane 1 is the molecular weight marker. Lanes 2-8 are the samples irradiated for 0, 3, 5, 15, 30, 60 and 120 min. The irradiation times are indicated below the lane numbers. Lane 2 corresponds to the solution without irradiation, and shows an RNaseA monomer band at 14 kDa and a dimer band at 28 kD, which resulted from the
impurity of the commercially available solution used. In Lanes 3-8, corresponding to the irradiated solutions, the dimer band intensities become clear with increasing irradiation time. In Lanes 5-8, trimer bands at 42 kDa can be observed, i.e. in samples exposed to more than 30 min of irradiation. These results show that light irradiation produces covalently bonded dimers and trimers, as observed in the lysozyme system.

**IV-3-4 Photochemically induced nucleation of Bovine pancreatic Ribonuclease A**

Photochemically induced nucleation of RNaseA was carried out. Our aim was to induce crystallization by photochemical perturbation where no spontaneous nucleation occurs. I arranged a metastable condition of supersaturation by changing both the protein and the precipitant concentration. As the precipitant, a mixed solution of NaCl and ammonium sulfate was used, in accordance with the report by Fedrov et al. Figure IV-10 (A)-(I) shows photographs of RNaseA droplets on a microbatch plate. The RNaseA concentrations were set to 15, 20 and 25 mg ml$^{-1}$. The respective precipitant solutions were 2.3 M NaCl and 1.44 M ammonium sulfate, 2.35 M NaCl and 1.47 M ammonium sulfate, and 2.4 M NaCl and 1.5 M ammonium sulfate. We made 3x3 matrixes of RNaseA and precipitant concentrations. The conditions are labeled (A) to (I) in Figure IV-10, and the concentrations of RNaseA and the precipitants in the droplets are indicated on the left side and below the photograph, respectively. Four simultaneous experiments for each condition were carried out. In conditions (A)-(E) and (G), no crystal appeared. In conditions (F) and (H), crystals appeared in 25% of droplets. In condition (H), crystals appeared in all droplets. These results show that experimental conditions (F), (H) and (I) induced spontaneous nucleation, whereas conditions (A)-(E) and (G) seem to have been metastable. In this latter condition, photochemically induced crystallization was expected to succeed. We tried to employ experimental condition
(E). Figure IV-11 shows photographs of RNaseA droplets exposed to light irradiation for 0 (a), 180 (b) and 300 s (c). No crystal was seen in the droplet without irradiation (a), and none in condition of Figure IV-10 (E), either. Crystals appeared in the irradiated droplets (b) and (c), and the number of crystals increased with increasing irradiation time. These results show that photochemically induced nucleation was succeeded in the RNaseA system lacking Trp residues.

**IV-4 Summary**

In this Chapter, it was investigated the mechanism of the photochemically induced nucleation of Bovine pancreatic ribonuclease A (RNaseA). In RNaseA system, photochemically induced nucleation brought about the photochemical reaction of the Tyr residual groups instead. The photochemical intermediate radical of the Tyr residue was observed in a transient absorption experiment. The radicals produced covalently bonded RNaseA dimers, which were observed by SDS-PAGE. The number of crystals exposed to UV light irradiation increased with increasing in irradiation time under to avoid spontaneously nucleated condition.
IV-5 References


Figure IV-1 Three dimensional structure of Bovine pancreatic ribonuclease A (PDB ID: 1FS3)
Figure IV-2 Molecular sequence of RNase A

KETAAAKFER QHMDSSTSA A
SSSNYNCNQMM KSRNLT KDRC
KPVNTFVHES LADVQAVCSQ
KNVACKNGQT NCYQSYSTMS
ITDCRETGSS KYPNCAYKTT
QANKHIIVAC EGNPYVPVHF
DASV
Scheme IV-1 Enzymatic reaction of RNase A
Figure IV-3 Irradiation setup for direct method
Figure IV-4 Transmission spectra of band pass filter UVTF-33U
Figure IV-5 Radiation spectra from 300 W Xe lamp (dashed line) through a 33U band pass filter (solid line) and through a monochromator at 280 nm (broken line).
Figure IV-6 Steady-state spectra of RNase A (a), Tyr (b) and Phe (c).
Figure IV-7 Transient absorption spectra of RNaseA (a), Tyr (b) and Phe (c).
Scheme IV-2 Photochemical mechanism of TyrOH

Excitation : TyrOH → TyrOH*

Ejection : TyrOH* → Tyr⁻ + e⁻ + H⁺

Deprotonation: Tyr⁻⁺ → Tyr⁻ + H⁺

Dimerization : 2Tyr⁻ → di-tyrosine
Figure IV-8 Conformation of covalently bonded di-tyrosine

Figure IV-9 Photograph of SDS-PAGE gel
Figure IV-10 Arrangement of metastable condition for PIN of RNase A
Figure IV-11 Photographs of droplets in PIN of RNaseA observed at 1 week after. Irradiation time was 0 (a), 180 (b) and 300 s (c).
Chapter V

Conclusion
V Conclusion

The studies made in Chapter II, III and IV, lead to conclusion that the covalently bonded dimer enhances nucleation of protein. Scheme V-1 shows the mechanism of photochemically induced nucleation of protein (a) and nucleation process (b).

In photochemical reaction of (a), protein is excited by the absorption of UV photon and an aromatic amino residue chromophore of protein produces the residual cation radical with ejecting an electron. The chromophore of lysozyme and RNaseA is Trp and Tyr. The cation radical produces neutral radical when protein solution is alkaline condition with pH value higher than pKa value of cation radical. Produced neutral radicals form the covalently bonded dimer of protein. Lysozyme involving neutral radical (residual Trp*) is denatured by further UV and/or Vis light irradiation. The ratio of increase in number of lysozyme crystals is proportional to that of the quantity of the covalently bonded dimer.

The enhancement mechanism by UV light irradiation is illustrated in Scheme V-1 (b). The two molecules (the smallest cluster) is unstable because two molecules bound to gather by a weak interaction (van der Waals or hydrogen bond). Thus, the two molecules cluster formation is rate determining step at the early stage of nucleation process. Whereas, if the covalently bonded dimer behaves similar to the smallest cluster (two molecule cluster), the dimer gathers protein molecules to form cluster consisting 3, 4, … molecules and finally grow to bulk crystal. Therefore, a nucleation starts from the dimer, nucleation frequency should be higher than the two molecule cluster.

The investigation of photochemically induced nucleation of protein would accelerate the protein structure determination by use of X-ray crystallography and therefore solve the bottleneck on the structure-based drug design.
Scheme V-1 The Mechanism of photochemically induced nucleation of protein (a) and nucleation process (b).
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