Chapter 4
Crystal Nucleation of Proteins Induced by Surface Plasmon Resonance

Tetsuo Okutsu

Abstract The crystallization of lysozyme and ribonucleaseA was induced using photochemical reactions triggered by surface plasmon resonance of gold nanostructures. The tryptophan residues of the protein are radicalized by the enhanced electric field induced by surface plasmon resonance. This radical reacts in the protein molecule to produce a reaction intermediate in which a nearby tyrosine residue is radicalized. This reaction intermediate reacts with another protein to form a dimer linked by tyrosine residues. Since this dimer is covalently bonded, it is stable without decomposition. With this as a nucleus, it grows into a crystal. An enhanced electric field induced by surface plasmon resonance of gold nanostructures was used to radicalize amino acids in proteins. Surface plasmon resonance induced by visible light radicalizes amino acids by the same mechanism as multiphoton absorption. When a metastable solution of lysozyme and ribonucleaseA was dropped on the substrate on which the gold nanostructure was constructed, and surface plasmon resonance of the gold nanostructure was induced, crystals precipitated.

Keywords Surface plasmon resonance · Protein crystallization · Lysozyme · RibonucleaseA · Gold nanostructure

4.1 Introduction

Experiments to crystallize proteins are important in the fields of drug discovery and structural biology. In the field of drug discovery, we elucidate the structure of target proteins that cause diseases. Based on the information of its structure, molecules that exactly fit the protein, i.e., drugs, are designed and synthesized. In the field of structural biology, studies are being made to elucidate functions from protein structures. In these fields, in order to determine the structure of a protein, X-ray crystallography is carried out after crystallizing the protein [1]. In Japan, synchrotron radiation facilities such as SPring-8 have been built, and the technology of crystal

T. Okutsu (✉)
Division of Molecular Science Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-cho, Kiryu-shi, Gunma-ken 376-8515, Japan
e-mail: okutsu@gunma-u.ac.jp

M. Sakamoto and H. Uekusa (eds.), *Advances in Organic Crystal Chemistry*,
https://doi.org/10.1007/978-981-15-5085-0_4

structure analysis has been advanced. At the same time, advances in science and technology to make crystals are desired.

Crystal growth of proteins is carried out by researchers in the field of drug discovery and biology using experience and intuition based on information in the literature and data base. On the other hand, basic research on crystal growth of proteins has been made from the viewpoint of crystal growth science regarding nucleation and growth mechanism [2]. In addition, researchers specializing in crystal growth have come into the business of crystallizing proteins for drug discovery [3].

There are several reasons why protein crystallization is more difficult than small-molecule substances. First, although proteins have large molecular weight, they aggregate with small intermolecular force such as van der Waals force and hydrogen bond, and therefore they have the property of being hard to crystallize. Protein is a colloidal particle with charge repulsion, and when it crystallizes, salting out cancels the charge and causes mild aggregation to crystallize. If the charge is roughly canceled, it will easily become an amorphous precipitate. Second, proteins have large molecular anisotropy and no symmetry. For this reason, there is a problem that when incorporated into the crystal phase, the crystal phase is entered only when approaching in a specific direction. Therefore, even in a supersaturation, small clusters are easily separated, so it is difficult to form a critical nucleus, and a supersaturated solution with a concentration many times that of the solubility still exists. Research to obtain protein crystals is to address these issues.

In recent years, as a method of crystal growth, a phenomenon that induces crystallization by the perturbation of light has been found and noted [4]. The action of light is roughly divided into physical perturbations and methods using chemical actions. Research using physical perturbations has been developed with a focus on groups in the United States and Osaka University. The Osaka University group has successfully commercialized protein crystallization as a venture business [5].

Our group has found the phenomenon of crystallization using chemical perturbation of light, and we have been working on elucidating its mechanism [6]. The crystallization of proteins induced by photochemical reactions is described. The appearance of the initial stage of crystal growth is shown in Fig. 4.1 by a model of ball gathering.

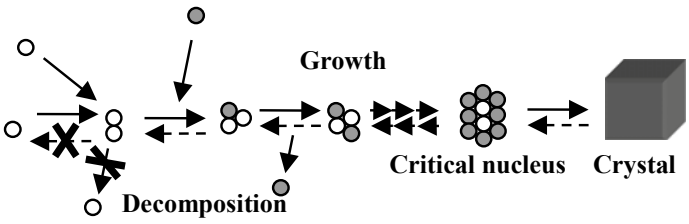

Fig. 4.1 Protein nucleation process and photoinduced crystallization mechanism. In the process of crystal nucleation, small aggregates below the critical nucleus are unstable and easily decomposed. Crystal nucleation is promoted by forming dimers that do not break covalent bonds through photochemical reactions

Two molecules meet and a bimolecular cluster is formed by intermolecular force due to hydrogen bonding or van der Waals force. Small clusters are unstable and do not grow spontaneously, but they grow spontaneously when they grow larger than the critical radius. In the case of a molecule such as a protein, even if the degree of supersaturation is large, the small clusters are often unstable and nucleation does not occur.

Imagine adding a stable bimolecular cluster bound covalently to the solution. The bimolecular cluster was the most unstable and easily dissociated, making it difficult to grow into a trimolecular cluster. However, if the bimolecular cluster is stable, it is easy to grow and a critical nucleus is easily formed. The protein crystallization method triggered by a photochemical reaction is to create a stable protein dimer in the system.

4.2 Photochemical Reaction of Proteins

Next, the relationship between the photochemical reaction of proteins and the mechanism of crystallization will be described. Figure 4.2 shows the chemical reactions that occur in proteins. First, the Trp residue absorbs light and becomes an excited state. At this time, a reaction intermediate in which the nitrogen atom on the five-membered ring is radicalized is generated [7, 8]. Then the radical undergoes a hydrogen abstraction reaction from the OH group of the neighboring Tyr residue, and the Tyr residue is radicalized. This radicalized protein has a long lifetime and reacts with other ground state proteins between collisions Tyr residues, and finally a protein dimer bound between Tyr–Tyr is formed [9].

The formation of this dimer can be observed by electrophoresis. The formation of the Tyr–Tyr bond can be confirmed by the characteristic fluorescence generated by the Tyr–Tyr bond. In hen egg-white lysozyme, dimers linked by Tyr^{53}–Tyr^{53} are generated. This dimer was found to resemble the arrangement of neighboring molecules in the crystal. It is thought that the dimer bonded at Tyr^{53}–Tyr^{53} grows into a crystal nucleus as a template. Nucleation by a similar mechanism has been shown using ribonucleaseA as a protein without thaumatin and Trp residues [10].

Fig. 4.2 Mechanism of photochemical reaction of protein. First, an excited state of tryptophan residue (Trp) is generated and radicalized. The radical transfer reaction proceeds in the molecule, and finally a reaction intermediate protein in which the tyrosine residue (Tyr) is radicalized is generated. This radical reacts to produce a protein dimer bonded between tyrosine, which functions as a template to grow into the crystal nucleus

In this study, we examined a method for inducing crystallization by photochemical reaction using a method that is not directly photoexcited amino acids of proteins [11, 12]. Protein was photoexcited using light of a wavelength at which the protein does not absorb. As a method for causing such photoexcitation, the reaction was induced with a reaction field based on a strong light-molecule coupling field constructed on a crystallization vessel. Proteins always have the problem of denaturation when exposed to ultraviolet light with light absorption. In order to examine the practical application of photoinduced crystallization, it is necessary to limit the amount of light to be irradiated to the minimum amount necessary for nucleation. In other words, it is necessary to carry out with as little light as possible. Excitation using a strong photo-molecular coupling field investigated in this study is equivalent to excitation by multiphoton absorption. Therefore, light absorption is a phenomenon that rarely occurs and excitation efficiency is extremely low. This is the same as using extremely weak light when excited by light absorption by one photon. The reaction field by the strong light-molecule coupling field was constructed in a commercially available crystallization vessel.

4.3 Reaction Field Using Light-Molecular Strong Coupling Field

The light-molecule strong coupling field used in this study is explained [13]. The light-molecule strong coupling field has a function of giving the influence of the electric field of light more strongly to the molecule. In this study, localized plasmon resonance was used as a strong light-molecule coupling field. In this field, chemical reaction of molecules was promoted. Brus et al. predicted that the reaction would theoretically be accelerated [14], Harris et al. reported that the aromatic photolysis reaction was promoted near the surface of silver nanoparticles [15].

Studies on reactions using multiphoton absorption by nonlinear optical phenomena using the electric field enhancement effect of plasmons are also progressing. For example, it is known that simultaneous two-photon absorption occurs when light having a high photon density. It has been also reported that simultaneous two-photon absorption is induced even when the plasmon-enhanced electric field by the steady light of the lamp [16, 17]. In this study, we investigated photoinduced crystallization that induces localized plasmon resonance by multiphoton absorption of visible light using gold nanostructures.

In this study, a checkerboard-like gold nanostructure and a gold-deposited film were used as a strong photo-molecular coupling field. Figure 4.3 shows a schematic diagram of the gold nanostructure. The gold nanostructure is a 100 nm × 100 nm, 40 nm-high gold nanostructure island aligned on a glass substrate at intervals of 200 nm. This structure was constructed in an area of 1 mm × 1 mm by electron beam drawing. Gold nanostructures were created in collaboration with a group of

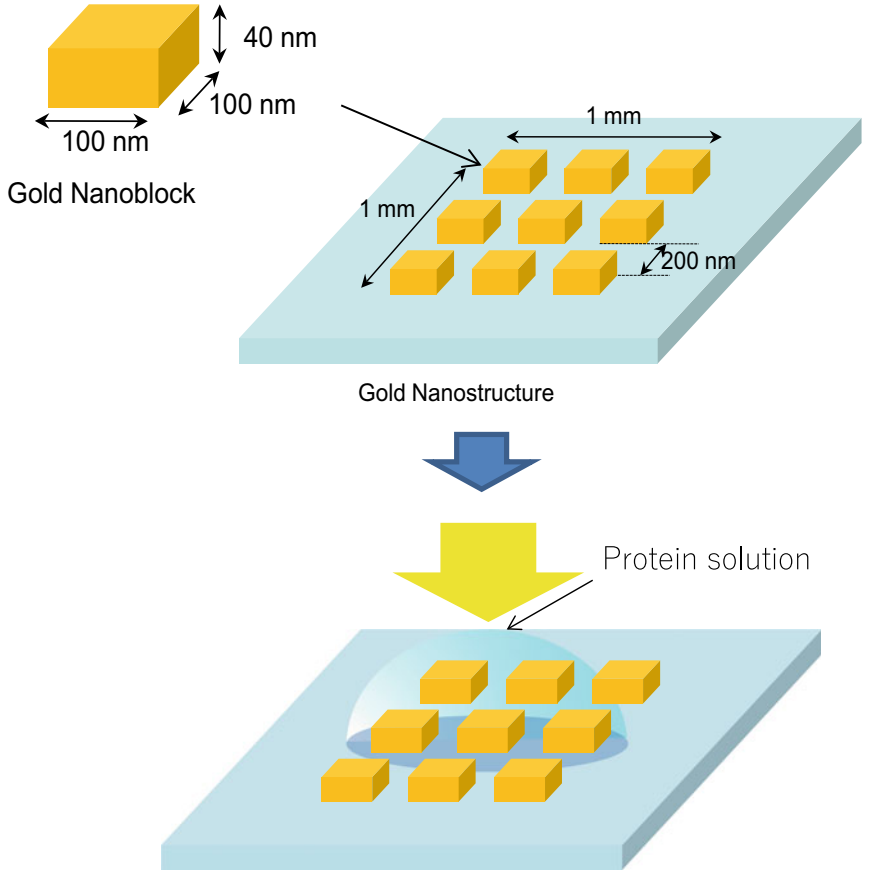

Fig. 4.3 Reaction field using plasmon resonance using photon-molecule strong coupling field with gold nanostructure

Prof. Hiroaki Misawa, Institute for Electrochemical Research, Hokkaido University. A 300 W Xe lamp was used as the irradiation light source.

4.4 Crystallization Experiment by Localized Plasmon Excitation

An experiment was carried out in which a protein solution was dropped on the gold nanostructure and irradiated with light. A supersaturated solution having a concentration three times the solubility was used as the protein solution. The crystallization experiment at this degree of supersaturation is at the boundary of whether or not

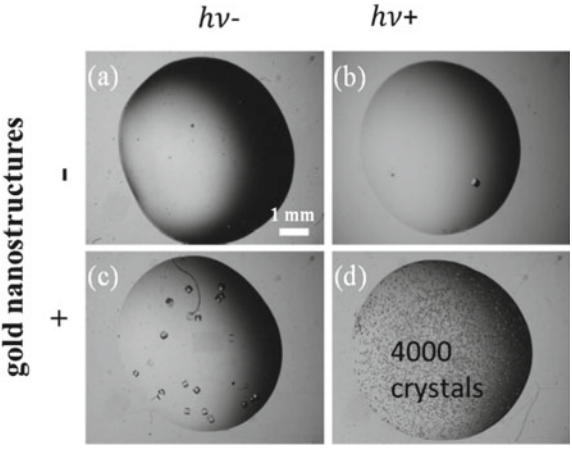

Fig. 4.4 Results of lysozyme crystallization experiment. Experimental results for the presence or absence of gold nanostructures and the presence or absence of light irradiation are shown. The appearance frequency of crystals increased remarkably in the presence of gold nanostructure and light irradiation

crystal nuclei appear spontaneously, and is a condition suitable for determining the effect of light-induced crystallization.

The presence or absence of gold nanostructures and the presence or absence of light irradiation on the protein solution on the substrate were compared. Figures 4.4a–d show photographs of the experimental results. Figures 4.4a–c are control experiments in which the protein solution was dropped onto a substrate without a gold nanostructure. (a) is without light irradiation and (c) is the result with light irradiation. No crystal appeared in (a), and one crystal appeared in (c). Since the solution used in this experiment is a solution having a boundary condition of "whether or not crystals appear spontaneously", variations occur in several appearance ranges depending on the droplets. (b) and (d) are experimental results on a substrate with a gold nanostructure. (b) shows the experimental results without light irradiation, and (d) shows the experimental results with light irradiation. In the experiment of (b), dozens of crystals appeared. This result can be said that the number of crystals appeared significantly increased compared to the experiments (a) and (c). In (d) about 4000 crystals appeared in the solution. Light-induced crystallization due to plasmon excitation of the gold nanostructure was confirmed. In the experiment of (b), the light of the Xe lamp was not applied, but the experiment was carried out under room light with a fluorescent lamp, and it is considered that crystals appeared due to the effect of the light of the fluorescent lamp, not the Xe lamp.

4.5 Reaction Field Construction and Crystallization Experiments with Gold-Sputtered Substrates

In the previous section, crystallization experiments were carried out using nanostructures. However, the construction of this nanostructure requires a long time. For example, it takes 20 h to create the 1 mm × 1 mm substrate shown in Fig. 4.3. It is

impossible for practical use to construct this nanostructure in a protein crystallization vessel. Localized plasmon resonance has been reported to occur on incompletely deposited metal films. In this study, we examined the effect of protein crystallization promotion by inducing plasmon resonance by thinly sputtered gold.

A sputtered gold film was thinly formed on a glass substrate. A gold sputtering apparatus for electron microscope (JEOL JFC-1500) used for preparation of SEM observation sample was used for the sputtered film preparation. The sample was prepared so that the thickness of the sputter was adjusted to 0–60 nm. Figure 4.5 shows the absorption spectrum of the sputtered film. An absorption peak appeared at 560 nm on the 10 nm thick substrate. This can be interpreted as that local plasmon resonance occurred because gold was in an island shape at the initial stage of sputtered deposition and had a structure similar to that of gold nanoparticles. As the thickness of the sputtered film was increased, the localized plasmon resonance at 560 nm disappeared, and broad absorption appeared at 500–1000 nm. This absorption is called gap mode plasmon resonance and is a characteristic spectrum of plasmon resonance caused by a film defect. Crystallization experiments were carried out with this substrate.

A protein solution was dropped using a crystallization vessel on which the gold thin film was sputtered, and visible light was irradiated. The crystallization experiment used a metastable state of supersaturation 3 ribonucleaseA solution. The experiment was carried out at a film thickness of 20 nm. The results are shown in Fig. 4.6. Crystals appeared under the condition of gold evaporation and light irradiation. From this result, we confirmed the effect of promoting crystallization when exciting localized plasmon in a thin and incompletely deposited film.

This method does not care about the problem of denaturation caused by excessive light and can obtain the effect of promoting crystallization without changing the experimental method conventionally carried out by researchers in the field of drug discovery. I think that it is useful.

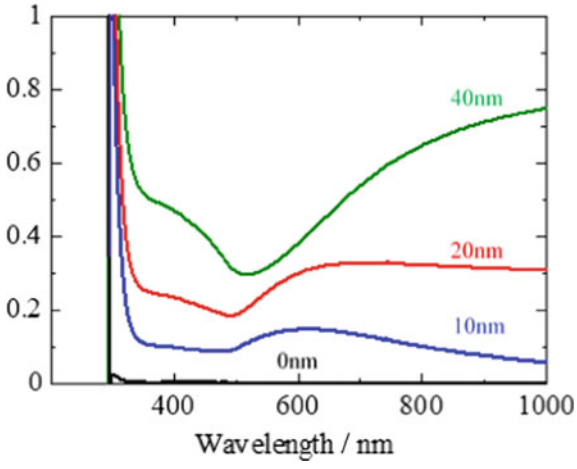

Fig. 4.5 Absorption spectrum of gold-sputtered thin film. The plasmon resonance of gold particles is visible at a film thickness of 10 nm, but gap mode absorption appears at a film thickness of 20 nm

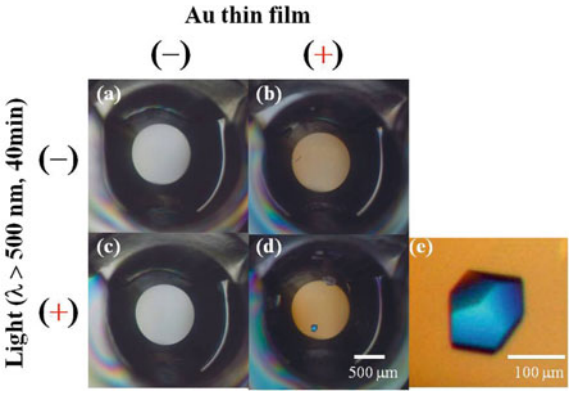

Fig. 4.6 Results of crystallization experiments on a sputtered gold substrate using ribonucleaseA. Crystals appeared under conditions of both gold sputtering and light irradiation

4.6 Future Prospective

Our recent experiments have found that crystallization is induced by a mechanism other than photochemical reactions. The enhanced electric field induced by surface plasmon resonance is said to have the same effect as optical tweezers. It has been suggested that the effect of optical tweezers may trap proteins and concentrate them locally, causing crystallization. We plan to study the phenomenon that protein crystallization is induced by this photophysical mechanism.

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