Journal of Photochemistry and Photobiology A: Chemistry 321 (2016) 180–186

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

Investigation of the mechanism of photochemically-induced lysozyme crystallization

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 26 January 2016
Accepted 27 January 2016
Available online 29 January 2016

Keywords:
Photo-induced crystallization
Lysozyme
Template

ABSTRACT

This work examined the mechanism involved in photoinduced crystallization of a lysozyme, focusing on the structure of the photoproducts that lead to crystal growth. It was determined that dimers formed via linking of tryptophan (Trp) residues are preferentially produced when applying a high-excitation photon fluence. However, these dimers do not lead to crystal growth. A low fluence forms dimers via the combination of tyrosine (Tyr) residues, generating six types of Tyr–Tyr dimers. The dimer that originates from the linking of Tyr^{53} and Tyr^{53} has a configuration similar to that of the two adjacent, yet non-linked, lysozyme molecules in the crystal unit cell. The Tyr^{53}–Tyr^{53} dimer hence acts as a template for lysozyme crystal nucleation.

1. Introduction

The crystallization of proteins is an important technique in genome-based drug discovery and further developments in this technology are expected. At present, the primary means of protein structural elucidation is X-ray crystallographic analysis, and the number of proteins analyzed has increased dramatically since synchrotron radiation instrumentation has been applied for this purpose. The present bottleneck is in fact the rate at which new protein crystals can be produced.

High-throughput protein crystallization is performed by large-scale screening processes, and various physical and chemical studies have been performed with the aim of finding methods to induce crystal formation from solution. The laser-induced method of crystallization, both discovered and developed over the past ten years, is of particular interest [1–4], and the application of this technique to the crystallization of proteins is currently an active research area. As an example, a group at Osaka University has reported that the emergence of protein crystals was promoted by irradiation of a protein solution with a femtosecond laser [5–7]. The effectiveness of this method is believed to be due to the alignment of molecules by the strong electric field associated with the laser light or from local condensation of solute molecules under strong photon pressure [8,9]. Methods of using laser-induced photon pressure to collect molecules at the light condensing point are also a topic of research [10].

Our own group has previously observed the photochemically-induced crystallization of lysozymes [11,12]. Herein, we describe the mechanism that we have so far elucidated to explain why crystals are readily formed when proteins are irradiated by electromagnetic radiation with wavelengths in the UV–vis region of the spectrum [13–17].

Previous studies by Adachi et al. [5] and ourselves [14] have demonstrated the presence of covalently bonded protein dimers in irradiated solutions. In the initial stage of crystal growth, small clusters are thermodynamically unstable and cannot grow into bulk crystals even if the solution is supersaturated. However, in the case that nucleation is initiated from stable bimolecular precursors that cannot dissociate due to their covalent linkage, then critical nuclei can be easily formed.

Empirical studies have shown that the onset of photoinduced crystallization depends on the light source employed. The use of a high-power light source does not result in protein crystallization, whereas irradiation with a weak continuous wave (cw) light source does induce crystallization. On the basis of such results, we believe that the photon fluence per unit time is important to the induction of crystallization. The excitation photon fluence is also thought to affect the structure of the photoproduct. Thus, it is possible that dimers generated by excitation with a high-fluence light source

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http://dx.doi.org/10.1016/j.jphotochem.2016.01.026
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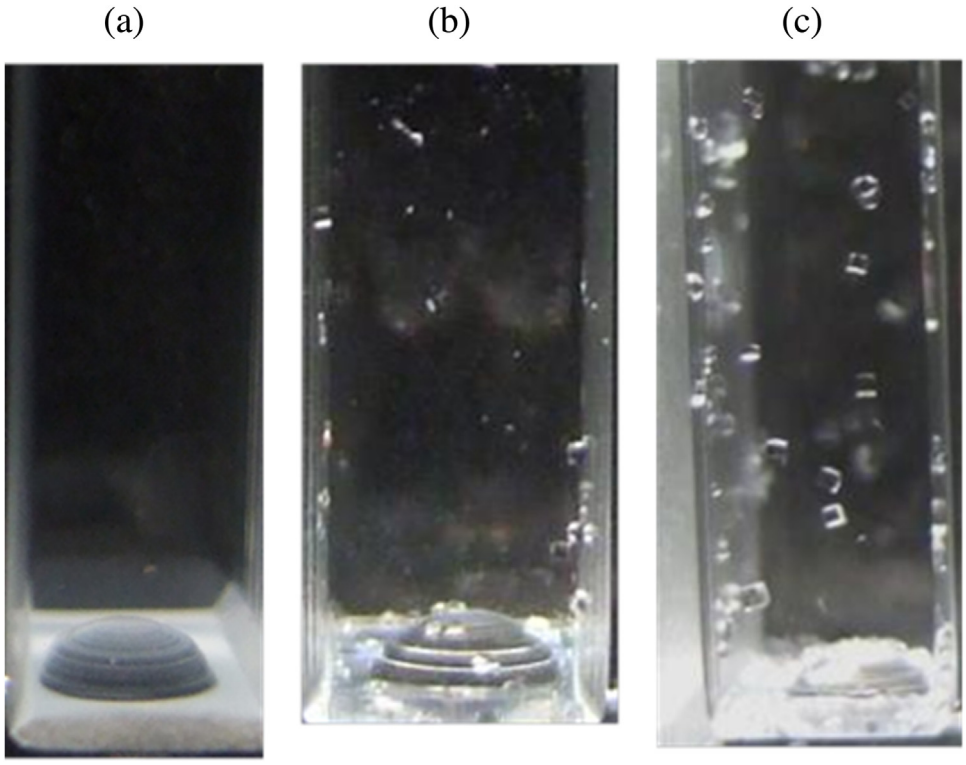

Fig. 1. Photographic images of protein solutions: (a) a non-irradiated control, (b) an irradiated solution, (c) a solution with pre-irradiated crystalline lysozyme.

may adopt structures that do not serve as nuclei, while dimers produced by irradiation with a low-fluence source will have a structure that readily grows into nuclei.

In this study, we examined the relationship between the structures of dimers that grow into crystal nuclei and the reaction mechanism involving intermediate radicals. We also assessed, at the amino acid level, whether or not templates are formed by the photochemical reaction of lysozymes in solution.

2. Experimental

Hen egg white lysozyme was purchased from Seikagaku (six times recrystallized, lot E02Z04) and was used without further purification. Sodium acetate, acetic acid and sodium chloride (NaCl), all GR grade, were purchased from Wako Pure Chemicals. The sodium acetate and acetic acid were dissolved in ultrapure water (Milli-Q) to produce a buffer solution (NaAc buffer, 50 mM, pH 4.3). This solution was centrifuged and filtered through a 0.45 μm single-use membrane (Millipore) prior to each experimental trial. Crystallization was performed under metastable conditions, typically with supersaturation ($C/C_e = 3$) and employing the batch method. The protein crystallization in this work followed a well-known procedure commonly used by biochemists and described in various textbooks [18,19].

A Xe lamp (150 W, USHIO) was employed as a light source for the light-induced crystallization trials. For transient absorption measurements, a Nd^{3+}: YAG laser (Tokyo Instruments Lotis II, 266 nm, pulse width 8 ns, 10 Hz) was used as the excitation light source. Details of the experimental setup for the transient absorption experiments have been provided previously in the literature [12]. Steady-state emission was recorded on a HITACHI F4500 fluorescence spectrometer and absorption spectra were recorded with a HITACHI U3300 spectrophotometer. The number of photons emitted by the Xe lamp was measured at the irradiated position with a spectroradiometer (USHIO USR 30).

3. Results and discussion

3.1. Template formation in crystals and solutions

In this study, we first generated templates and conducted experiments to confirm the growth of crystal nuclei. Templates were obtained by irradiating protein crystals with light, assuming that dimers generated by the reaction between adjacent molecules in the crystal would act as templates for crystal formation. In contrast, photochemically-induced dimers resulting from reactions in solution can occur at various sites, and so the position of the amino acid that reacts is not limited. Therefore, not all the dimers in the solution will have a structure that functions as a template. For this reason, we predicted that the template generation efficiency would vary between cases in which dimers were generated by reactions in a crystal or in a solution.

Fig. 1(a) shows the results obtained from a control trial in which we dissolved untreated crystals in a buffer solution, added a precipitant, and allowed the solution to stand for 24 h. Crystals did not emerge from this solution because the concentration was low. Fig. 1(b) presents an image from an experiment in which we irradiated a solution of the lysozyme and then added a precipitant. Approximately 20 crystals were formed in this solution. Finally, a trial was conducted in which crystals were first irradiated and then dissolved in the buffer along with the precipitant. The result is shown in Fig. 1(c). The number of crystals generated in this case was approximately four times greater than the number obtained in the trial in which the protein solution was irradiated (Fig. 1(b)). This result demonstrates that products generated in the solid state can serve as templates, and suggests that a greater number of templates suitable for crystal formation were present in the last trial. It is considered that the crystalline products have the same conformation as the two adjacent molecules in the initial dimer, and that these dimers thus grow to form nuclei that serve as templates in the supersaturated solution.