Dehydration Process of Protein Crystals by Micro-Brillouin Scattering

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Polymorphism and dehydration process have been studied by the micro-Brillouin scattering technique in hen egg white lysozyme crystals without cross-linking. Two types of crystal with tetragonal and monoclinic systems have been successfully grown by the two-liquid interface method. The dehydration processes of tetragonal and monoclinic crystals have been investigated by the exposure of crystals to open air. Sound velocity increases markedly owing to the increase in intermolecular interaction between lysozyme molecules, while the attenuation of sound wave decreases markedly owing to the decrease in friction generated by mobile water. The time dependences of sound velocity in two crystals have been discussed on the basis of the Avrami–Erofe’ev model. It is found that a monoclinic crystal dehydrates much faster than a tetragonal one.

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1. Introduction

The hydration mechanism plays an important role in sustaining the three-dimensional structure and biological activity of protein molecules both in solution and in crystals. Protein crystals are grown by the solution-growth technique. The effects of water molecules on protein crystals have been focused on protein–water interactions in the crystal lattice. Protein crystals include 30–70% (w/w) water. When a crystal is exposed to open air, intracrystalline water gradually evaporates and a crystal changes into a so-called air-dried crystal. Therefore, crystals are generally preserved in the growth solution. Air-dried crystals were intensively studied by Vijayan and colleagues.9–11) The researchers explored the hydration and related mobility of proteins. They discussed the water-mediated transformation caused by the humidity reduction in the environment. The effects of water on protein crystals were also studied from the physicochemical viewpoint, focusing on fundamental crystal properties including hydration and Young modulus.4–6) These studies on elastic properties used protein crystals that were cross-linked by glutaraldehyde. The cross-linking can produce significant effects on intermolecular interaction in the crystal lattice. We thus studied the dehydration process of a protein crystal without cross-linking.

Micro-Brillouin spectroscopy has been a reliable method of noncontact micro probing for elastic properties.7,8) Elastic properties provide information on the microscopic interaction of proteins and were studied by several investigators.9,10) Brillouin scattering measurement enables us to determine sound velocity, elastic constant, and damping of sound waves. It is also known to be a powerful tool to study the elastic properties in a high-frequency gigahertz range and a wide temperature range. Furthermore, it requires only a small volume of a sample to be studied. Therefore, it is quite suitable for very small materials.

Recently, the temperature dependence of elastic properties in lysozyme crystals has been investigated by Brillouin scattering.11) The relative humidity response in a lysozyme crystal was also studied by Brillouin scattering.12,13) These works, however, did not show any time response of the Brillouin spectrum of a hen egg white lysozyme (HEWL) crystal. Therefore, in the present study, the time response of acoustic properties of HEWL crystals during dehydration was studied by micro-Brillouin scattering. The time evolution of the dehydration process was discussed on the basis of the Avrami–Erofe’ev model.

2. Experimental Procedure

The experimental setup of a micro-Brillouin scattering instrument is shown in Fig. 1. The Brillouin scattering spectra were measured at the backward scattering geometry. A Sandercock-type 3 + 3 pass tandem Fabry–Perot interferometer (FPI) was combined with an optical microscope and operated to acquire the frequency spectra of scattered light. The Brillouin spectra were measured in the ±25 GHz frequency range with a free spectral range of 30 GHz. The sample temperature was controlled within ±0.1°C using a cryogenic cell (LINKAM HTMS600).

Tetragonal HEWL crystals were grown by the two-liquid interface method that employing an insoluble dense liquid.14) Some crystals were grown on the interface of two liquids of lysozyme solution and a dense liquid such as fluorinate. The crystals were grown using 25 mg/ml lysozyme and 5% (w/v) NaCl in 50 mM acetate buffer solution (pH = 4.5) at 25°C. All crystals show the crystal habits of [110] and [101] planes. The polarizing micrograph of an as-grown crystal is shown in Fig. 2(a). The laser beam was incident perpendicular to the [110] habit plane, which was the favored growth crystallographic plane of an as-grown crystal. Monoclinic HEWL crystals were grown also by the two-liquid interface method. The crystals were grown using 45 mg/ml lysozyme and 3% (w/v) NaNO3 in 50 mM acetate buffer solution (pH = 4.5) at 25°C. The polarizing micrograph of an as-grown crystal is shown in Fig. 2(b).

3. Results and Discussion

The observed Brillouin spectrum of a tetragonal HEWL crystal shows one broad longitudinal acoustic (LA) mode, as shown in Fig. 3. The dehydration process was examined under the condition that a protein crystal is exposed to open air. Brillouin shift increases and full width at half maximum (FWHM) gradually becomes narrow when the dehydration progresses. The Brillouin shift \( \nu = qV_L/2\pi \) and FWHM, \( \Gamma \) were determined from the spectra. In the present study, the longitudinal sound velocity \( V_L \) was calculated from the Brillouin shift and the scattering wave vector \( q = 2\pi n\sin(\theta/2)/\lambda \), where \( n \), \( \lambda \), and \( \theta \) are the refractive
index of the sample, the wavelength of the laser, and the scattering angle, respectively. The refractive index value of a HEWL crystal reported in the literature was used. The preliminary experiment shows that the refractive index of a dehydration HEWL crystal is nearly equal to that obtained under wet conditions using the refractive index free Brillouin scattering geometry. The LA mode attenuation coefficient of $C_11$ is related to FWHM of the Brillouin component as shown in the equation,

$$\frac{C_{11}}{C_0} = \frac{V}{L}.$$

The time dependence of $V_L$ of a tetragonal HEWL crystal at a constant temperature is plotted as shown in Fig. 4. At 40°C, the sound velocity markedly increases after 20 min, and approaches the horizontal asymptote. In contrast, sound velocity at 30°C moderately increases and reaches a constant value that is slightly lower than that at 40°C. On the other hand, the time dependence of attenuation of a tetragonal HEWL crystal at a constant temperature weakens.
The types of water in protein crystals were qualitatively classified into two on the basis of its behavior: one is mobile water among protein molecules and the other is immobile water strongly bound to protein molecules.\textsuperscript{16,17} Both types of water can exist in a crystal immediately after exposure to open air. The former will easily be evaporated from the crystal, while the latter can still remain in the crystal. With this consideration, Fig. 4 shows that almost all mobile water can be evaporated when the crystal is exposed to open air. Thus, the change in the mobile water content in the crystal affects the sound propagation studied in this work. The decrease in mobile water content may increase the force constant between lysozyme molecules and a marked increase in sound velocity occurs. It may cause also the decrease in friction generated by mobile water; therefore, the sound attenuation decreases remarkably.

The dehydration kinetics can be explained by using various kinetics equations, which have the best application in solid-state reactions. Several kinetics equations or models have been developed, including growth controlled reactions, phase-boundary-controlled reactions, diffusion-controlled reactions, power-law equations, and equations based on the order of reactions.\textsuperscript{18,19} We found that the dehydration process of tetragonal HEWL crystals obeys the Avrami–Erofe’ev type reaction (AE model), known as the contracting area equation.

\begin{equation}
\ln(1 - r(t)) = k^m t^m, \tag{1}
\end{equation}

where $k$ is the dehydration rate constant, $t$ is the time, $m$ is the dimension number of reaction, and $r$ is the normalized dehydration fraction, given by

\begin{equation}
r = \frac{V(t) - V(0)}{V(\infty) - V(0)}, \tag{2}
\end{equation}

where $V(t)$ is the sound velocity at the time $t$. With the increase in temperature, the dimension of this process changes from a slow two-dimensional process to a fast three-dimensional process in accordance with the AE model. In the mechanism that leads to a phase-boundary-controlled reaction, it is assumed that the nucleation step occurs virtually instantaneously, so that the surface of each particle is covered with a product layer. Nucleation of the reactant, however, may be a random process, not followed by rapid surface growth. As nuclei grow larger they must eventually impinge on one another, so that growth ceases where they touch each other. This process has been considered by Avrami and Erofe’ev.\textsuperscript{20–23}

Regarding tetragonal HEWL crystals, it is clear that the behavior of time dependence is different between 30 and 40 °C. Figure 5(a) shows the plot of $r$ as a function of time during the dehydration at 30 and 40 °C. For a monoclinic HEWL crystal, Fig. 5(b) shows the plot of $r$ as a function of time during the dehydration at 32 °C. This process is already three-dimensional even at 32 °C. The half-life of the dehydration process of a tetragonal crystal is about four times longer than that of a monoclinic one ($t_{1/2}^{\text{mono}} < t_{1/2}^{\text{tetra}}$), as shown in Fig. 5. It is found that a monoclinic crystal dehydrates much faster than a tetragonal one. This finding indicates that the crystal structure is very sensitive to dehydration rate.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig5.png}
\caption{(a) Normalized fraction of anhydrous tetragonal HEWL crystals at 30 and 40 °C and (b) monoclinic HEWL crystal at 32 °C. The curves of $r$ are fitted using the Avrami–Erofe’ev model of solid-state reaction. The open symbols indicate the experimental data and the solid lines are the curves.}
\end{figure}

4. Conclusions
We have studied polymorphism and dehydration process in lysozyme crystals without cross-linking. Tetragonal and monoclinic HEWL crystals have been successfully grown by the two-liquid interface method. By the micro-Brillouin scattering method, we have observed the time evolution of sound velocity and attenuation in tetragonal and monoclinic HEWL crystals. In the dehydration process of tetragonal HEWL crystals, at about 40 °C, the sound velocity markedly increases, while that at 30 °C moderately increases. The increase in sound velocity can be caused by the enhancement of the intermolecular interaction between lysozyme molecules. On the other hand, the time evolution of attenuation at a certain temperature decreases owing to the decrease in friction generated by mobile water. It is found that a monoclinic crystal dehydrates much faster than a tetragonal one. This finding indicates that the protein–water interaction is very sensitive to the dehydration rate and the rate depends on the crystal lattice structure. It is found that the dehydration process of HEWL crystals obeys the Avrami–Erofe’ev model approximately. Further experiments are necessary to clarify the mechanism underlying the dehydration process.

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1) Sudarsanakumar and M. Vijayan: Acta Crystallogr., Sect. D 51 (1995) 390.
2) H. G. Nagendra, N. Sukumar, and M. Vijayan: Proteins: Struct. Funct. Genet. 32 (1998) 229.
3) N. Sukumar, B. K. Biswal, and M. Vijayan: Acta Crystallogr., Sect. D 55 (1999) 934.
4) V. N. Morozov and T. Y. Morozova: Biopolymers 20 (1981) 451.
5) V. N. Morozov, T. Y. Morozova, G. S. Kachalova, and E. T. Myachin: Int. J. Biol. Macromol. 10 (1988) 329.
6) V. N. Morozov and T. Y. Morozova: J. Biomol. Struct. Dyn. 11 (1993) 459.
7) F. Jiang and S. Kojima: Appl. Phys. Lett. 77 (2000) 1271.
8) Y. Ike, Y. Matsuda, S. Kojima, and M. Kodama: Jpn. J. Appl. Phys. 45 (2006) 4474.
9) M. Tachibana, H. Koizumi, and K. Kojima: Phys. Rev. E 69 (2004) 051921.
10) H. Koizumi, M. Tachibana, and K. Kojima: Phys. Rev. E 73 (2006) 041910.
11) A. V. Svanidze, S. G. Lushnikov, and S. Kojima: JETP Lett. 84 (2006) 551.
12) S. Speziale, F. Jiang, C. L. Caylor, S. Kriminski, C.-S. Zha, R. E. Thorne, and T. S. Duffy: Biophys. J. 85 (2003) 3202.
13) C. L. Caylor, S. Speziale, S. Kriminski, T. Duffy, C.-S. Zha, and R. E. Thorne: J. Cryst. Growth 232 (2001) 498.
14) H. Adachi, T. Watanabe, M. Yoshimura, Y. Mori, and T. Sasaki: Jpn. J. Appl. Phys. 41 (2002) L726.
15) B. Ceruelle, F. Cesbron, J. Berthou, and P. Jolles: Acta Crystallogr., Sect. A 30 (1974) 645.
16) B. W. Matthews: J. Mol. Biol. 33 (1968) 491.
17) G. Otting, E. Liepinsh, and K. Wuthrich: Science 254 (1991) 974.
18) H.-B. Liu and X.-C. Zhang: Chem. Phys. Lett. 429 (2006) 229.
19) J. H. Sharp, G. W. Brindley, and B. N. Narahari Achar: J. Am. Ceram. Soc. 49 (1966) 379.
20) M. Avrami: J. Chem. Phys. 7 (1939) 1103.
21) M. Avrami: J. Chem. Phys. 8 (1940) 212.
22) M. Avrami: J. Chem. Phys. 9 (1941) 177.
23) B. V. Erofe’ev: C. R. Acad. Sci. URSS 52 (1946) 511.