Effect of intracrystalline water on longitudinal sound velocity in tetragonal hen-egg-white lysozyme crystals

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Longitudinal sound velocity of tetragonal hen-egg-white (HEW) lysozyme crystals was measured during air drying by ultrasonic pulseecho method. The sound velocity increases with exposure to open air and approaches a constant value. The maximum value is ~2900 m/s that is about 1.6 times as much as that of original one before drying. In addition, the sound velocity clearly recovers to original one after immersing the dried crystal in solution. Therefore, the sound velocity in tetragonal HEW lysozyme crystals can be reversibly changed due to dehydration and rehydration. These changes in sound velocity are discussed in the light of water-mediated intramolecular and intermolecular interactions in the crystals.

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I. INTRODUCTION

Water plays a central role in maintaining the structure and activity of protein molecules both in solution and in the crystal. The effects of intracrystalline water on protein crystals have been extensively studied from a crystallographer's viewpoint, focusing on changes in lattice structure, in molecular conformation, and in the arrangement of surrounding water molecules [1–3]. The effects of intracrystalline water on protein crystals have been also investigated from the physicochemical viewpoint, focusing on fundamental crystal properties including hydration and Young modulus [4–6]. However, almost all the latter studies have used protein crystals which were cross linked by glutaraldehyde. The cross linking can produce significant effects on intermolecular interactions between proteins. In addition, it can deteriorate the quality of protein crystals. Therefore, the studies on physical properties of protein crystals without cross linking are desirable for the understanding of intrinsic role of water in the crystals.

To measure intrinsic physical properties, high quality protein crystals of sufficient size for the measurements are required. We have obtained tetragonal hen-egg-white (HEW) lysozyme crystals of millimeter size by a salt-concentration gradient method originally proposed by Ataka and Katsura [7]. The high quality of as-grown crystals was characterized by x-ray topography [8,9]. This success in obtaining large protein crystals with high quality has led to the measurements of mechanical properties, such as Vickers microhardness [10] and sound velocity [11]. Especially, the sound velocity in protein crystals is related to elastic constants which reflect the intramolecular and intermolecular interactions in the crystals.

In this paper, we report the longitudinal sound velocity of tetragonal HEW lysozyme crystals without cross linking measured during air drying by ultrasonic pulseecho method. It is shown that the sound velocity increases with exposure to open air and approaches a constant value. The maximum value is observed to be ~2900 m/s which is about 1.6 times as much as that of original one before drying. Moreover, it is shown that the sound velocity clearly recovers original one after immersing the dried crystal into solution. Therefore, the sound velocity can be reversibly changed due to dehydration and rehydration. These changes in sound velocity are discussed in the light of water-mediated intramolecular and intermolecular interactions in the crystals.

II. EXPERIMENT

Tetragonal HEW lysozyme crystals were grown by a salt-concentration gradient method at 23°C in test tubes held vertically and using $NiCl_2$ as a precipitant [12]. Large crystals up to a size of 4 mm were grown over two weeks. Almost all the crystals had growth habits such as {110} and {101} crystallographic faces. The crystals were kept in the growth solution before the measurements of sound velocity.

Protein crystals include a large amount of water [13]. The water can be easily evaporated when the crystals are exposed to open air. Consequently, the crystals can be dehydrated. In this work, to examine the effect of intracrystalline water on protein crystals, the sound velocity of tetragonal HEW lysozyme crystals was measured during air drying, i.e., as a function of exposure time to open air under a relative humidity of less than 75% and a temperature of about 22°C.

The measurements of sound velocity were carried out using the pulseecho mode operation of the ultrasonic pulser/receiver (PR35, JSR Ultrasonics). A longitudinal ultrasonic transducer capable of operating at a frequency of 10 MHz was used in pulse transmission and echo receiving. The larger (110) habit face of tetragonal HEW lysozyme crystals was used for the measurements of sound velocity. The (110) face of as-grown crystals was placed on the flat head of the transducer held vertically in open air. The longitudinal ultrasonic wave was introduced along [110] direction in the crystals by the transducer. The traveling time Δt of the longitudinal ultrasonic wave, spent passing through the crystal, is measured as the time difference between reflective echos from the bottom and top faces of the crystals. The traveling distance is twice as much as the thickness W of the crystal.

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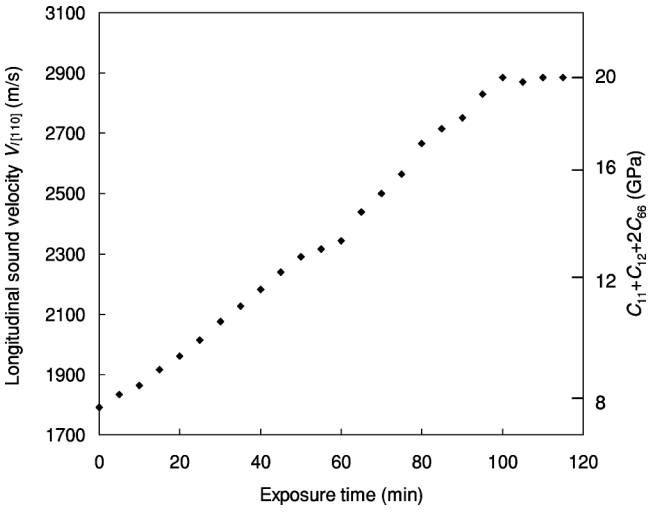

FIG. 1. Change in longitudinal sound velocity $V_{l[110]}$ along [110] direction in a tetragonal HEW lysozyme crystal as a function of exposure time to open air. The scale of composite elastic constant, $C_{11}+C_{12}+2C_{66}$, corresponding to the longitudinal sound velocity $V_{l[110]}$ is denoted as ordinate on right in the figure.

The crystal thickness was measured with a micrometer only after the successive measurements of the traveling time as a function of exposure time to open air. The sound velocity was estimated by dividing $2W$ by Δt, assuming that no change in crystal dimensions occurs in the measurements.

To confirm the drying of the crystals, the weight of the crystals was measured as a function of exposure time to open air. In addition, the change in lattice constants during air drying was also observed by x-ray diffraction.

III. RESULTS AND DISCUSSION

Figure 1 shows the longitudinal sound velocity $V_{l[110]}$ along [110] direction in a tetragonal HEW lysozyme crystal as a function of exposure time to open air. As shown in Fig. 1, the sound velocity before drying is in good agreement with 1817 m/s of as-grown tetragonal HEW lysozyme crystals in solutions reported previously [11]. The sound velocity increased with increasing exposure time and approached a constant value. The change in sound velocity included several stages. The maximum value of the sound velocity was ~2900 m/s. This value is about 1.6 times as much as that of original one before drying. Thus, the sound velocity of the tetragonal HEW lysozyme crystal increased by ~60% due to air drying.

The longitudinal sound velocity $V_{l[110]}$ along [110] direction in tetragonal structure with 422 space group is related to the elastic stiffness constant C_{ij} by the following equation:

$$C_{11} + C_{12} + 2C_{66} = 2\rho V_{l[110]}^2,$$

where ρ is the mass density of the crystal. In tetragonal HEW lysozyme crystals, $\rho = 1.21$ Mg/m^3 [11]. Therefore, the scale of composite elastic constant, $C_{11}+C_{12}+2C_{66}$, corresponding to the longitudinal sound velocity $V_{l[110]}$, was also denoted as ordinate in right in Fig. 1. Assuming the isotropic body, the longitudinal sound velocity V_l is related to the Young modulus E by

$$E = \frac{(1+\sigma)(1-2\sigma)\rho V_l^2}{1-\sigma},$$

where σ is the Poisson ratio. The corresponding other elastic constants such as shear modulus μ and bulk modulus K are given by $\mu = E/2(1+\sigma)$ and $K = E/3(1-2\sigma)$, respectively. These elastic constants in protein crystals can be evaluated with $\sigma = 0.33$ in most polymers [11]. The elastic constants of dried tetragonal HEW lysozyme crystals were estimated with $V_l = V_{l[110]} = 2900$ m/s, which is the maximum longitudinal sound velocity along [110] direction in the dried crystal in Fig. 1. The estimated values of elastic constants are summarized in Table I with those for $V_l = V_{l[110]} = 1817$ m/s in as-grown tetragonal HEW lysozyme crystals in solutions reported previously [11].

Almost all the studies on elastic properties of protein crystals, except for cross-linked crystals, have been carried out in solutions or wet conditions. The longitudinal sound velocities of ribonuclease-A and human hemoglobin crystals in solutions were found to be 1784 m/s and 1828 m/s, respectively, using a laser-generated ultrasound [14]. The composite elastic constant, $C_{11}+C_{12}+2C_{66}$, of tetragonal HEW lysozyme crystals in wet conditions was also found to be between 6.2 and 12.6 GPa, using Brillouin scattering [15]. In addition, the Young modulus of triclinic HEW lysozyme crystals in solutions was found to be between 0.1 and 0.5 GPa, using triple-point bending [6]. These values of elastic properties, except for the Young modulus, are in good agreement with our previous results for as-grown tetragonal HEW lysozyme crystals in solutions measured by ultrasonic pulseecho method with a transducer in Table I.

As presented in Table I, the change in elastic properties due to air drying was shown, to our knowledge, for the first time in protein crystals without cross linking. For example, the Young modulus of tetragonal HEW lysozyme crystals

TABLE I. Longitudinal sound velocity $V_{l[110]}$ along [110] direction in tetragonal HEW lysozyme crystals before and after air drying, and the corresponding elastic constants such as composite elastic constant, $C_{11}+C_{12}+2C_{66}$, Young modulus E, shear modulus μ, and bulk modulus K. The details of these evaluations are described in the text.

	$V_{l[110]}$ (m/s)	$C_{11}+C_{12}+2C_{66}$ (GPa)	E (GPa)	μ (GPa)	K (GPa)
As-grown crystals in solution	1817	7.99	2.70	1.02	2.65
Dried crystals	2900	20.4	6.87	2.58	6.74

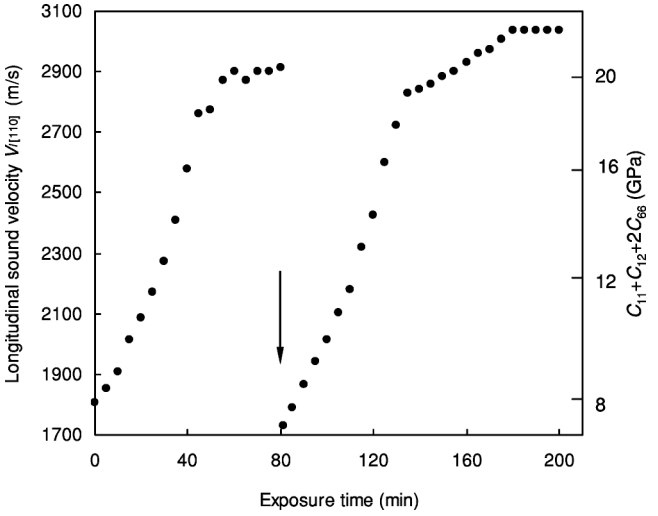

FIG. 2. Reversible change in longitudinal sound velocity $V_{l[110]}$ along [110] direction in a tetragonal HEW lysozyme crystal as a function of exposure time to open air. The time, at which the dried crystal was immersed in solution, is indicated by an arrow.

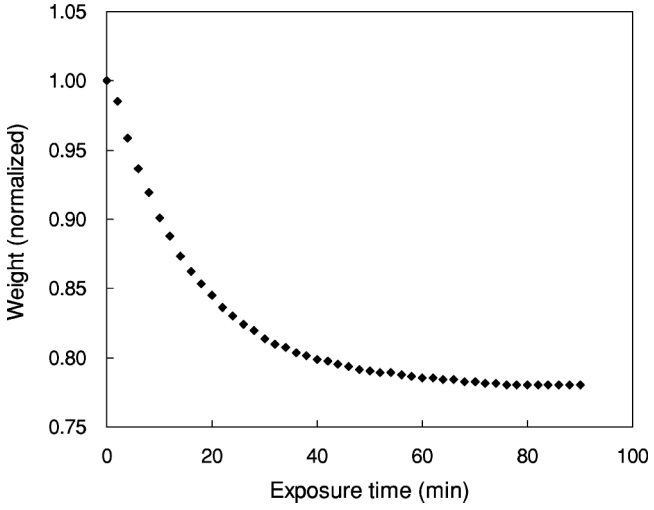

FIG. 3. Change in weight of a tetragonal HEW lysozyme crystal as a function of exposure time to open air. The ordinate is normalized by the weight of the crystal before drying.

drastically increases due to air drying, as shown in Table I. The maximum Young modulus of 6.87 GPa in the dried crystal is about 2.5 times as much as 2.70 GPa of as-grown crystals in solutions. For a reference, we also mention the relative humidity, H, dependence of the Young modulus in cross-linked tetragonal HEW lysozyme crystals measured by vibrating reed method previously [4]. The Young modulus also increased with decreasing relative humidity, or drying. The Young modulus at $H=60\%$ was about 3.5 times as much as that at $H=90\%$. These results show that the change in Young modulus of tetragonal HEW lysozyme crystals without cross linking due to air drying is not as much as that of cross-linked ones.

It is interesting to observe if the change in sound velocity is reversible or not. To examine the reversibility, the sound velocity of a tetragonal HEW lysozyme crystal was measured as a function of exposure time to open air. As shown in Fig. 2, after the sound velocity reached the maximum, the dried crystal was immersed in solution. Consequently, the sound velocity clearly recovered to the original one before drying. Then, the sound velocity increased with increasing exposure time again. These results indicate that the sound velocity is reversibly changed by the dehydration and rehydration. Thus, there is a type of water in the crystal, which can freely move in and out of the crystal, and the water strongly affects the sound velocity. This also suggests that common protein crystals including a large amount of water are a kind of porous material with holes of nanometer size.

The behavior of water in protein crystals was qualitatively classified into two types: one is mobile water among protein molecules and the other is immobile water strongly bound on proteins [13,16]. Both types of water will be present in the crystal just after exposure to open air. The former will be easily evaporated from the crystal while the latter can be still left in the crystal.

To examine the amount of evaporated water, the weight of a tetragonal HEW lysozyme crystal was measured as a function of exposure time to open air. As shown in Fig. 3, the weight decreased with increasing exposure time and finally, 22% weight of the crystal was lost. Assuming that all of the lost weight is the evaporated water, the amount of evaporated water can be estimated to be 6.4×10^{-20} g/unit cell. In this estimation, it was assumed that, before drying, the unit cell is $a=b=79.1$ Å, $c=37.8$ Å, and $Z=8$, and 39% of the crystal volume is occupied by water[17] and the mass density of water is 1.0 g/cm^3. On the other hand, the remained water in the crystal can be estimated to be 2.9×10^{-20} g/unit cell.

According to a neutron crystallographic study with tetragonal HEW lysozyme crystals grown by a salt-concentration gradient method similar to this work, 157 bound water molecules were observed on a lysozyme molecule [18]. The corresponding weight of bound water can be estimated as 3.8×10^{-20} g/unit cell. This value is in moderate agreement with 2.9×10^{-20} g/unit cell of remained water measured in this work. This means that almost all mobile water can be evaporated when the crystal is exposed to open air. Thus, the change in mobile water content in the crystal affects the sound velocity measured in this work.

Figure 4 shows the change in lattice constant a ($=b$) of tetragonal HEW lysozyme crystal as a function of exposure time to open air. The lattice constant decreased with increasing exposure time and approached a constant value. The change in the lattice constant included some stages. Consequently the lattice constant shrank by ~2.3%. When the dried crystal is immersed into solution, the lattice constant clearly recovered the original value. Similar change was also observed in c axis although the maximum shrinkage of c axis was slightly larger than that of a ($=b$) axis. Thus, the lattice constants in tetragonal HEW lysozyme crystals are reversibly changed by the dehydration and rehydration. Such changes in lattice constants have been also observed by other groups [1–3].

From the comparison of Figs. 1 and 4, the decrease of the lattice constant in tetragonal HEW lysozyme crystals seems to be correlated with the increase of the sound velocity.

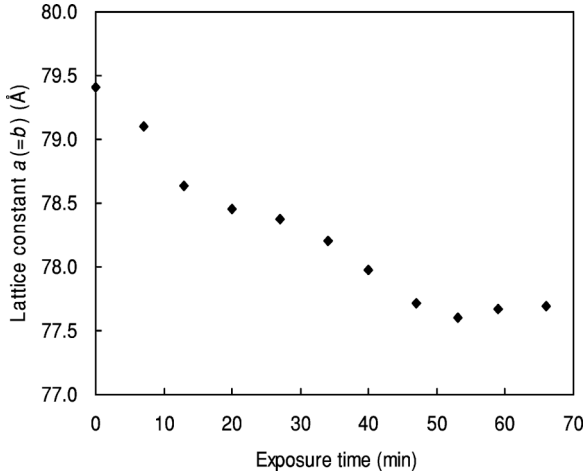

FIG. 4. Change in lattice parameters a (=b) of a tetragonal HEW lysozyme crystal as a function of exposure time to open air.

However, the maximum shrinkage of ~2.3% in the lattice constant, as seen in Fig. 4, is much smaller than the increase of ~60% in the sound velocity, as seen in Fig. 1, estimated assuming that no shrinkage of lattice constants occurs. Thus, the significant increase of the sound velocity cannot be simply related to the shrinkage of the lattice constant.

The sound velocity in protein crystals is related to the intermolecular interactions in the crystals. The intermolecular interactions in protein crystals can be understood by the macrobond approach in which a macrobond contact is defined if at least one atom pair whose distance is less than 4 Å exists between neighboring molecules with bound water molecules [19]. In tetragonal HEW lysozyme crystals before drying, there are four kinds of macrobond contacts between lysozyme molecules [20]. As shown in Fig. 4, the air drying for the crystals decreased the lattice constant. This will increase the number and area of the macrobond contact of lysozyme-lysozyme. The increase of such intermolecular contacts will contribute to the increase of the sound velocity due to air drying.

The strength of intermolecular contacts in protein crystals can be estimated by the macrobond approach. It is also applied to dried protein crystals when the structures are known. In fact, the macrobond energy has been calculated for a partially dried monoclinic HEW lysozyme crystal [20]. This suggests that the macrobond approach is a potential method to know a possible change in the intermolecular interaction between wet and dried crystals. Thus, the contribution of intermolecular interactions to the increase of sound velocity would be estimated by the macrobond approach.

The sound velocity in protein crystals might be related to not only intermolecular but also intramolecular interactions, since volumes of protein molecules are much large compared with those of common organic (small) molecules. In general, it is difficult to directly measure the intramolecular interaction, e.g., the elasticity of a protein molecule. The elasticity of hydrated protein molecules, i.e., protein molecules with bound water, in the crystal is considered to be similar to that in solution. The compressibility of hydrated protein molecules in solution has been obtained by measuring sound velocity of protein solutions [21,22]. The isothermal compressibility β_T is related to the bulk modulus K with $1/\beta_T = K$. With the isothermal compressibility of 7.73×10^{-11} m^2/N in the hydrated lysozyme at 25°C reported previously [23], the bulk modulus of hydrated lysozyme is estimated to be 12.9 GPa. This value is significantly large compared with 6.74 GPa in dried crystals, as well as 2.65 GPa in as-grown crystals in solution. Thus, it seems that the large bulk modulus of hydrated lysozyme molecules is not predominant in that of the crystals.

More recently, the apparent sound velocity of hydrated lysozyme molecules in solutions was obtained to be 1958 m/s by measuring sound velocity of lysozyme solutions [24]. This value is slightly larger than 1817 m/s of as-grown crystals in solution but much smaller than ~2900 m/s of dried crystals. The relatively small apparent sound velocity of hydrated lysozyme molecules seems to be in contradiction with the large bulk modulus as mentioned above. However, the small apparent sound velocity might be due to the weak intermolecular contact between lysozyme molecule and bound water, rather than lysozyme molecule itself. Thus, the increase of the strength of lysozyme-bound water contact also would be required for the increase of sound velocity due to air drying. From these results, it is suggested that the removal of mobile water due to air drying would increase the strength of lysozyme-bound water contact, as well as that of lysozyme-lysozyme contact as mentioned above. In addition, several stages in the change in sound velocity as seen in Figs. 1 and 2 might reflect the formation of new intermolecular contacts in lysozyme-lysozyme and lysozyme-bound water during air drying.

IV. CONCLUSION

We have shown that the sound velocity in tetragonal HEW lysozyme crystals without cross linking drastically increases due to air drying. In addition, it has been shown that the sound velocity is reversibly changed by dehydration and rehydration. Such changes in sound velocity can be attributed to the changes in intermolecular contacts of lysozyme-lysozyme and lysozyme-bound water in the crystals. However, the details of the changes, e.g., increasing rate, of the sound velocity, depend on sample temperature, relative humidity, size of the samples, crystal quality, and so on. Further measurements under the control of temperature and humidity are required for more detailed understanding of water-mediated intramolecular and intermolecular interactions in protein crystals.

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