Solubility measurements of protein crystals under high pressure by \textit{in situ} observation of steps on crystal surfaces

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\textbf{Abstract.} We successfully measured equilibrium temperatures $T_e$ of tetragonal hen egg white lysozyme crystals under high pressure by \textit{in situ} observation of steps on the \{110\} faces of the crystals. The dependence of $T_e$ on the concentration of lysozyme corresponds to that of the solubility $C_e$ on temperature. The precision of the solubility determined in this study is significantly higher than that in previous works. One $T_e$ could be measured during short time less than 70 minutes. This method for solubility measurements of protein crystals under high pressure is the fastest one at this stage.

1. Introduction

Solubility measurements of protein crystals under high pressure are useful for studying correlation between pressure and a three-dimensional (3D) structure of a protein molecule. Solubility data of crystals under high pressure is indispensable for preparing crystals under high pressure. If we can perform X-ray structure analysis of a protein crystal under high pressure, and if reversible deformation of a protein molecule under high pressure is observed, the correlation between pressure and the 3D structure can be studied systematically. Such the correlation will be utilized for understanding some deepsea organisms in detail \cite{1}. In addition, the correlation is probably beneficial for studying correlation between a 3D structure and a function of a molecule, since effects of pressure on an enzymatic activity of a protein \cite{2, 3} are probably due to the change in a 3D structure of a protein under high pressure.

Although there are a few studies on protein solubilities under high pressure \cite{4, 5}, most of them took long time for the measurements, and their precision is relatively low. Therefore, we need faster and more sensitive methods for the solubility measurements under high pressure. At this moment, Sazaki \textit{et al.} performed the fastest measurement of equilibrium temperatures of protein crystals under high pressure with highest precision \cite{6}. They measured changes in concentration distribution around crystals by two-beam interferometry. However, errors of their data were still large for precise thermodynamical analyses.
To improve the precision of the solubility measurements, we focused our attention on the in situ observation of step motion on a crystal surface. Since the observation of step motion is much more sensitive than that of a concentration distribution in detecting equilibrium conditions as described elsewhere [7], we have measured the solubility of tetragonal lysozyme crystals under high pressure by in situ observation of steps.

2. Experimental procedures

Tetragonal crystals of hen egg-white lysozyme were grown from solutions containing six times recrystallized lysozyme (Seikagaku Kogyo Co. Ltd.). We used lysozyme without further purification. All other chemicals were of reagent grade. Lysozyme was dissolved in a 50mM sodium acetate buffer (pH 4.5). A sodium chloride solution of 50 mgmL\(^{-1}\) was prepared in the same 50mM acetate buffer. A supersaturated solution was prepared by mixing equal volumes of the lysozyme solution and the sodium chloride solution. The supersaturated solution (lysozyme: 150 mgmL\(^{-1}\)) was transferred into an in situ observation cell, and was incubated at 20.0˚C for a few hours to prepare suitable seed crystals in the cell. The observation cell was made of a glass capillary and sapphire window of 1mm thickness. The capillary (1 mm inner diameter × 7 mm height) was adhered onto the window. About 5–10 crystals (~100 \(\mu\)m in size) were nucleated separately on the window. All the crystals were prepared under atmospheric pressure. Before each measurement of the equilibrium temperature \(T_e\), we replaced the solution in the cell with fresh one.

A laser confocal microscope combined with a differential interference contrast microscope (LCM-DIM) [8] (OLYMPUS, FV300, IX71) and an objective (OLYMPUS, SLCPlanFl 40x) were used for the step observation. In situ observation under high pressures was conducted by using a high-pressure vessel (Syn corporation Ltd., PC-100-MS) with the observation cell (Figure 1). We observed steps on an upper surface of a lysozyme crystal through the sapphire window and the crystal itself.

From the observation of the steps on the crystal surface, we determined \(T_e\) of the crystal. When the crystal and the solution were almost equilibrated, the step was neither receding nor advancing. When the temperature of the sample was set lower than its \(T_e\), the step advanced. On the other hand, when the temperature was raised higher than its \(T_e\), the step receded. Therefore, \(T_e\) was defined as the average of the lowest temperature at which the step receded (\(T_d\)) and the highest temperature at which the step advanced (\(T_g\)) within 10 minute. The error of \(T_e\) (\(\delta T_e\)) is defined as the half of the temperature difference between \(T_d\) and \(T_g\).

This method has more advantages than interferometry. For instance, we need not a vacuum-deposited gold mirror on the bottom of an observation cell to enhance reflectivity of light. In addition, we don’t have to optimize optical lengths of the cell and the crystal size.

3. Results and discussion

3.1. Determination of an equilibrium temperature

Figure 2 shows examples of photomicrographs of the observed two-dimensional (2D) islands. When the temperature of the sample was raised higher than its \(T_e\), the step around the islands receded,
and the islands shrunk, as shown in Figure 2 (a). On the other hand, when the temperature was set lower than its $T_e$, the step around the islands advanced, and the islands expanded and hence coalesced each other (Figure 2 (b)).

Figure 2. Changes in the positions of the steps on the {110} faces of tetragonal crystals. (a) When the crystal dissolved, the steps receded. (b) When the crystal grew, the steps advanced.

Figure 3. An example of a determination process of an equilibrium temperature ($C=30.5 \text{ mgm}^{-1}$). The solid line shows the temperature change. Crystals apparently dissolved at ● and grew at ■. As the temperature of the cell approached $T_e$, the value of $\delta T_e$ approached zero.

Figure 4. Temperature dependence of the solubility. Closed symbols and solid curves indicate the results of this study, and open symbols and broken curves show those taken from the works of Sazaki et al [6, 9]. Circles show the solubilities under 0.1 MPa, and squares show those under 50 MPa.
approached zero. In this example, the equilibrium temperature should lie between 21.4 ($T_g$) and 22.4°C ($T_d$). Since it is not clear that the growth and dissolution of lysozyme crystals proceed symmetrically or not, we show our results with error bars, and temporarily determined the equilibrium temperature as $22.0 \pm 0.5^\circ C$. All the equilibrium temperatures were determined within 70 minutes. In principle, the longer we take time for the measurement, the higher we can improve the precision of the measurement.

3.2. Solubility curves

Figure 4 shows solubility curves. Closed symbols indicate the results of this study, and open symbols show those taken from the works of Sazaki et al [6, 9]. Circles show the solubilities under 0.1 MPa, and squares show those under 50 MPa. Our data well correspond to Sazaki's interferometric ones. In addition, the precision of our method is significantly higher than that of the interferometric ones, in particular in a low temperature range.

Table 1 shows some comparisons between our method and the interferometric one. Our method enabled to measure the solubility of the crystal significantly faster (70 min.) than the interferometric one (180 min.). This is probably due to higher sensitivity of our method. With the interferometric method, fringe shifts of 10% of the interval between each fringe were detected. The shifts correspond to the change in the solution concentration of 1.96 mgmL$^{-1}$. And the shifts appear over about 100μm solution layer adjacent to a crystal face. On the other hand, we can detect much less concentration change. For example, if we assume that (1) steps sweep all over a crystal face, and one layer of the crystal face completely dissolves, and (2) all the dissolved molecules stay in the 100μm solution layer adjacent to the crystal face, the concentration in the layer increases by 0.003 mgmL$^{-1}$. However, the islands shown in Figure 2 were probably surrounded by macrosteps. At this stage, we have not observed elementary steps under high pressure. Thus, macrosteps should be considered. For instance, even in the case that one hundred layers sweep all over a crystal face, the solution concentration increases by 0.3mgmL$^{-1}$. This change is still less than that of the interferometric method. Since we could observe the slight change in the step position, the precision of $T_e$ should be even higher than expected above. In addition, we have successfully observed elementary steps (7.2nm height) of in the glucose isomerase crystals under high pressure through a sapphire window of 1mm thickness [10]. Therefore we believe that we will observe the elementary step of lysozyme crystal of 5.6nm height, and obtain more precise $T_e$.

| Observation object | Our method | Sazaki’s method [6] |
|---------------------|------------|---------------------|
| Microscopy          | LCM-DIM    | Two-beam interferometry |
| Objective           | 40×        | 5×                  |
| Time                | 70 min.    | 180 min.            |

4. Conclusions

We developed a novel technique to determine a solubility curve under high pressure by in situ observation of steps on protein crystals using an optical high-pressure vessel. In this study, we successfully demonstrated the usefulness of our technique using tetragonal lysozyme crystals. LCM-DIM was used for the observation of the steps on the {110} faces to judge whether they were growing or dissolving. By observing the step motion of the crystals we could determine the equilibrium
temperature of the crystals in the solution at a given protein concentration within 70 min. The precision of the solubility in this study is significantly higher than that in previous works.

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**References**
[1] Yayanos A A 1986 *Proc. Natl. Acad. Sci. USA* **83** 9542
[2] Makimoto S, Suzuki K and Taniguchi Y 1984 *J. Phys. Chem.* **88** 6021
[3] Mozhaev V V, Lange R, Kudryashova E V and Balny C 1996 *Biotech. Bioeng.* **52** 320
[4] Takano K J, Harigae H, Kawamura Y and Ataka M 1997 *J. Cryst. Growth* **171** 554
[5] Suzuki Y, Sawada T, Miyashita S, Komatsu H, Sazaki G and Nakada T 2000 *J. Cryst. Growth* **209** 1018
[6] Sazaki G, Nagatoshi Y, Suzuki Y, Durbin S D, Miyashita S, Nakada T and Komatsu H 1999 *J. Cryst. Growth* **196** 204
[7] Van Driessche A E S, Gavira J A, Lopez L D P and Otalora F 2009 *J. Cryst. Growth* **311** 3479
[8] Sazaki G, Matsui T, Tsukamoto K, Usami N, Ujihara T, Fujiwara K and Nakajima K 2004 *J. Cryst. Growth* **262** 536
[9] Sazaki G, Kurihara K, Nakada T, Miyashita S and Komatsu H 1996 *J. Cryst. Growth* **169** 355
[10] Sazaki G, Suzuki Y, Matsumoto M, Nagasawa M, Nakajima K and Tamura K *Cryst. Growth Des.* submitted