Rapid solubility measurement of protein crystals as a function of precipitant concentration with micro-dialysis cell and two-beam interferometer

Katsuyoshi Nakazato, Tomoko Homma and Tatsuya Tomo

Department of Applied Physics, College of Humanities and Sciences, Nihon University,3-25-40 Sakurajosui, Setagaya-ku, Tokyo, 156-8550, Japan.
E-mail: nakazato@ phys.chs.nihon-u.ac.jp

A novel dialysis-based technique was developed for the rapid and sample-saving determination of the precipitant dependence of protein solubility. We developed a unique thin dialysis cell which permits both the direct optical measurement of protein concentration and the observation of concentration gradients around a crystal via two-beam interferometry. Interferograms around a lysozyme crystal under the desired concentrations of precipitant (NaCl) and lysozyme were observed to determine whether the crystal is growing or dissolving. This technique enables the precipitant dependent solubility curve of lysozyme crystal to be obtained in two days using a small sample (total amount: 7 mg lysozyme).

Keywords: protein solubility, precipitant, dialysis, interferometry.

1. Introduction

The crystallization of protein in three-dimensional structural analysis of protein molecules presents a bottleneck. One of the most important parameters of crystallization is protein solubility, which determines the driving force for nucleation and growth processes. The solubility of a protein crystal depends on several factors, such as temperature, the type and concentration of precipitants and pH. A substantial number of protein crystals have been prepared by reducing the solubility of the protein through the use of precipitants such as various salts or PEGs. Therefore, the determination of the dependence of solubility curves on precipitant concentration is very important. However, the classical method requires a large amount of sample. In addition, establishing a solubility curve requires a great deal of time (Ataka, M. & Tanaka, S.(1986); Ataka, M. & Asai, J.(1988)), because the growth rate of protein crystals is much lower than that of small molecules. Usually, the equilibration process using classical techniques requires several weeks. Moreover, during this period, the protein must be stabilized in the desired state and the system must be maintained uncontaminated during repeated sampling.

The present study was performed with the intention of shortening the time required to obtain a solubility curve of protein crystal as a function of precipitant concentration and to reduce the amount of protein used for solubility measurements. Solubility of protein under a given temperature and pH depend on the concentration of the protein and that of the precipitant. Accordingly, the solubility of protein crystal can be determined if these two parameters can be measured at the state in which the crystal and solution reach equilibrium. In the present study, a unique thin dialysis cell was developed which is suitable for the rapid change of precipitant concentration in a protein solution and the direct optical measurement of protein concentration therein. The proposed technique applies two-beam interferometry to observe concentration gradients around a crystal in the dialysis cell in order to determine whether the crystal is growing or dissolving (Komatsu *et al*.,1993; Sazaki *et al.*, 1996). In the present paper, we show that the combination of a micro-dialysis cell and an interferometer is useful for both shortening the time required to obtain a solubility curve of protein crystal as a function of precipitant concentration and reducing the amount of protein required for analysis.

2. Experimental procedure

2.1. Sample preparation

Six-times-recrystallized hen egg-white lysozyme (Seikagaku Kogyo Co. Ltd.) was used without further purification. The other chemicals used for preparing solution were of reagent grade. Lysozyme was dissolved into a 50 mM sodium acetate buffer (pH 4.5) to a final concentration of 200 mg/ml. The protein concentration was optically analyzed using an absorption coefficient α at 280 nm = 2.64 ml/mg cm (A.J.Sophianopoulos *et al.*, 1962). Tetoragonal lysozyme crystals were prepared via the hanging drop method with a reservoir solution containing 5% NaCl and 50 mM sodium acetate (pH 4.5) at 18˚C.

2.2. Dialysis cell

Using quartz plates and acryl blocks, we constructed a thin dialysis cell (inner cell) for the incorporation of a sample solution and a crystal. Figures 1a and b show the cross sectional view and the top view of the inner cell, respectively. Bottom plate A shown in Fig.1a was jointed to upper plate B via two small glass spacers having a thickness of 0.3 mm. To minimize the volume of a sample solution in the inner cell, a thin quartz plate (thickness: 0.3 mm) was used as the bottom plate (A in Fig.1a). Half of the bottom plate of the inner cell was vacuum deposited with gold in

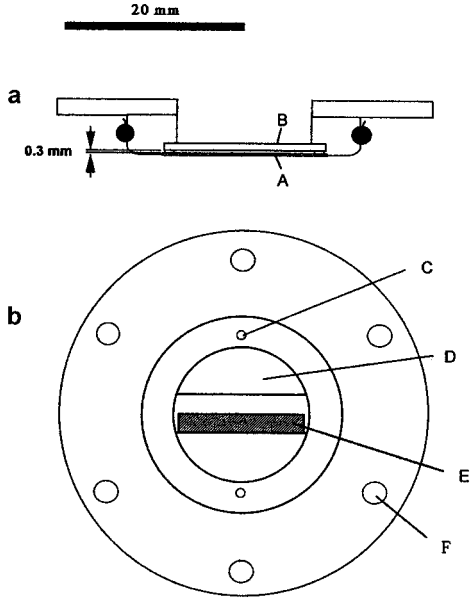

Figure 1
A cross sectional view of the inner cell (a) and a top view of the inner cell (b). A: bottom plate, B: upper plate, C: pipe to supply sample solution, D: dialysis membrane, E: bottom plate deposited with gold, F : hole to joint the inner cell to the outer cell.

order to enhance the reflectivity of light for interferometry and the other half was used for the optical measurement of protein concentration. A dialysis membrane Spectra/Pore MWCO 2000 was adhered closely to the bottom plate of the inner cell with an O ring. The volume of the inner cell covered with a dialysis membrane was 70 µl. The outer cell to incorporate precipitant solution was also constructed of a quartz plate and acryl blocks. For the optical concentration measurement of protein in the inner cell, a quartz plate was set at the bottom of the outer cell. The outer cell was jointed to the inner cell via six bolts by placing silicon rubber between the two cells. The inner volume of the outer cell attached to the inner cell was 3.8 ml.

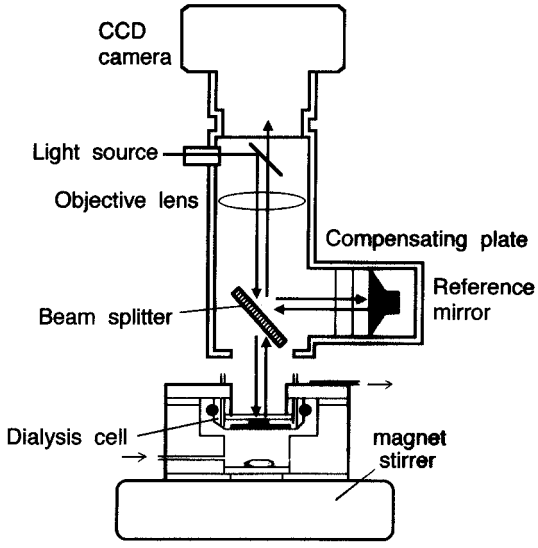

Figure 2
Michelson interferometer and the dialysis cell Allows show the flow direction of dialysis solution.

2.3. Solubility measurements

One lysozyme crystal was placed on the bottom plate in the inner cell (see Figs.1 and 2) with an aliquot of reservoir solution used to prepare the crystal. An aliquot of solution containing 100 mg lysozyme/ml, 0.15 M NaCl and 50 mM sodium acetate (pH4.5) was supplied into the inner cell through the slender stainless pipe attached to the inner cell. The inner cell was quickly jointed to the outer cell which had been filled with a solution containing 0.15 M NaCl and 50 mM sodium acetate (pH 4.5). The exchange of the precipitant concentration in the inner cell was carried out by supplying a solution containing an appropriate concentration of precipitant to the outer cell using a liquid chromatography system (BioRad Biologic system). The solution in the outer cell was gently stirred using a magnetic stirrer in order to enhance the passage of precipitant through the dialysis membrane. The conductivity of the solution passed through the outer cell was monitored via the system to check whether the concentration of precipitant (NaCl) in the outer cell reaches a desired concentration.

In order to minimize the amount of protein, a number of solubility measurements were carried out by decreasing the protein concentration in the inner cell step-by-step from 98.0 mg/ml to 5.1 mg/ml. After the solubility measurement under the highest concentration of lysozyme, the concentration of protein in the inner cell was slightly reduced by injecting a small volume of solution containing an appropriate concentration of NaCl and 50 mM sodium acetate (pH 4.5) through the pipe attached to the inner cell. At each stage, the concentration of protein in the dialysis cell was optically measured using a spectrometer Shimadzu UV1600.

The observation of the concentration gradients around a crystal to determine whether the crystal is growing or dissolving was carried out using a Michelson interferometer (Model M Plan 2.5 TI/0.075, Nikon) attached to an optical microscope of reflection type. The sample was illuminated using a 5 mW He-Ne laser (Melles Griot). Interferograms were recorded using a CCD camera (CAMEDIA C-3040, Olympus). All measurements were performed at 20˚C.

3. Results and discussion

3.1. Performance of the dialysis cell

Figure 3 shows the relationship between the concentration of lysozyme in the dialysis cell and the absorbace at 281.5, 295 or 300 nm. As expected from the absorption coefficient of lysozyme and the optical pass length of the inner cell (0.3 mm), the absorbance at 281.5 nm (peak wavelength of lysozyme) did not show a linear increase over lysozyme concentrations above approximately 20 mg/ml. In the present study, the concentration of lysozyme in the dialysis cell was measured at 300 nm, since the absorbance at the wavelength increased linearly up to 100 mg lysozyme/ml as shown in Fig.3. It should be noted that the dialysis membrane used (Spectra/Pore MWCO 2000) has a considerable absorption, 0.35 at 300 nm. However, this absorption did not hinder the optical concentration measurement of lysozyme as the total absorbance, including the dialysis membrane, did not exceed 1.6, even at 100 mg lysozyme/ml.

In the present study, the concentration of precipitant in the inner dialysis cell was changed by supplying a desired concentration of precipitant into the outer dialysis cell. Therefore, it is necessary to examine the time required to equilibrate the concentration of precipitant in the inner cell to that in the outer cell. For this purpose, the passage of NaCl from the inner cell to the outer cell, through the dialysis membrane, was examined by measuring the conductivity of solution in the inner cell. The test revealed that sufficient equilibration of precipitant concentrations in the two cells occurs within approximately 30 minutes. Furthermore, we determined the time required to homogenize the lysozyme concentration in the inner cell after the injection of an aliquot of buffer solution to the

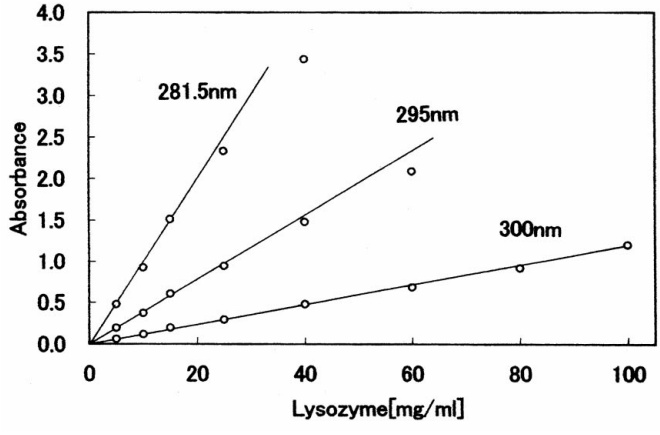

Figure 3
The relationship between the concentration of lysozyme in the dialysis cell and the absorbance.

inner cell. By monitoring the absorbance change of protein solution in the dialysis cell, we estimated this time to be approximately 50 minutes. The changes of interferograms during these treatments also supported above results. Therefore, we observed interferograms around a crystal after one hour in all cases in which the concentration of precipitant and(or) protein in the inner cell was changed forcibly.

3.2. Solubility of hen egg-white lysozyme

We determined the equilibrium concentration of precipitant (NaCl) surrounding a tetragonal lysozyme crystal under a constant concentration of lysozyme by observing the curving of the interference fringes at 20˚C. Figure 4 shows the results for the case in which the concentration of lysozyme was set to 40.2 mg/ml. The crystal and the solution were nearly equilibrated at 0.36 M NaCl, since the fringes are straight lines, as shown in Fig.4a. If the concentration of NaCl was set above the equilibrium concentration of the precipitant, the crystal began to grow, and the concentration of lysozyme molecules around the crystal decreased. This led to the decrease in the refractive index and bending of the interference fringes in the vicinity of the crystals, as shown in Fig. 4b.

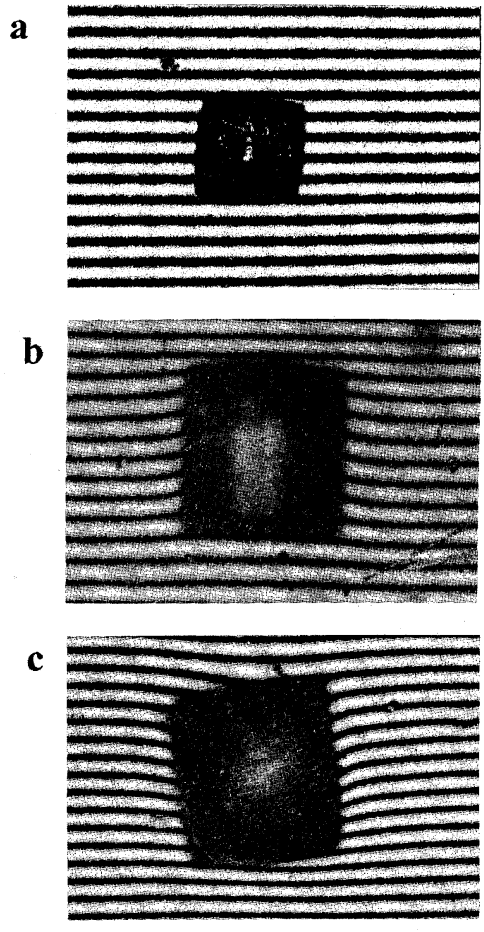

Figure 4
Interferograms around the tetragonal crystal of lysozyme. Conditions: 40.2 mg lysozyme/ml in 50 mM sodium acetate buffer (pH4.5) at 20˚C. (a) Equilibrium (0.36 M NaCl); (b) growth (0.40 M NaCl); (c) dissolution (0.30 M NaCl).

On the other hand, when the concentration of NaCl was reduced below the equilibrium concentration of the precipitant, the crystals began to dissolve and the fringes were bent toward the opposite direction (Fig.4c).

Figure 5 shows the solubility curve of the lysozyme crystal obtained as a function of NaCl concentration at 20˚C. The positions of open circles and solid circles represent the conditions under which lysozyme crystals dissolved and grew, respectively. Half solid circles show the conditions under which the crystal and the solution were nearly equilibrated. Since this technique depends on how accurately we judge the straightness of the fringes, fringe shift was evaluated using the way of Sazaki *et al*. (1996). In the present study, a fringe shift of 10% of the interval between each fringe could be readily detected. In order to elucidate the equilibrium concentration of NaCl at a constant concentration of lysozyme, the concentration of precipitant at which the fringe shift value becomes 0 was determined by bracketing the fringe shift values nearest 0 at dissolution and the growth conditions. The open triangles in Fig.5 indicate the precipitant concentrations determined using this method. The errors in determining the solubility were found to depend on the precipitant dependence of the solubility of lysozyme. As shown in Fig. 5, the errors in measuring the equilibrium concentration of precipitant decreased with the increase of the slope of the solubility curve in terms of the concentration of precipitant. This is likely due to the drastic change in the conditions surrounding the crystal to either the supersaturated region or the undersaturated region, which occurs upon a very small increment or decrement of precipitant concentration from equilibrium concentration. Consequently, the errors at higher concentration of precipitant become large. Unfortunately, we could not directly compare the values obtained in the present study with values obtained previously in this laboratory using a conventional method, because the temperature was 3˚C higher than 20˚C, although the same lysozyme was used. The solubility curve obtained at 20˚C was considerably shifted to lower concentrations of NaCl than that at 23˚C. The solubility value at 0.43 M NaCl and at 20˚C was consistent with the value reported by Sazaki *et al*. (1996) who measured the temperature dependence of solubility of

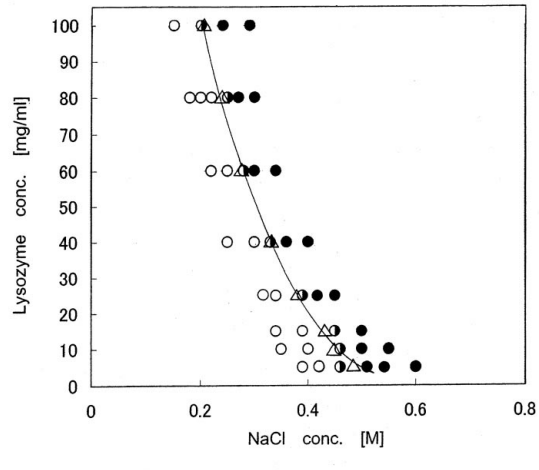

Figure 5
Solubility curve of tetragonal lysozyme crystal as a function of NaCl concentration at 20˚C. Circles represent the conditions where a tetragonal lysozyme crystal apparently dissolved (open circles) and grew (solid circles). Open triangles represent the equilibrium conditions determined by bracketing the fringe shift values nearest 0 at the dissolution and the growth.

tetragonal crystals of the same lysozyme (Seikagaku Kogyo Co.Ltd.) with two-beam interferometry.

This technique was designed to shorten the time required to obtain a solubility curve of protein crystal as a function of precipitant concentration. We succeeded in drastically shortening the measurement time. The results of the present study were obtained in two days when all measurements were performed continuously. Our technique is several tens times faster than the conventional method; the classical method requires at least several weeks to establish a solubility curve. Furthermore, the amount of protein was significantly reduced by using a thin micro-dialysis cell which is suitable for (1) the direct optical concentration measurements of protein, (2) the observation of concentration gradient around a crystal with two beam interferometer and (3) the change of the concentration of precipitant and protein. The total amount of lysozyme required for this work was only about 7 mg. It is easy to greatly reduce the sample amount by decreasing the optical pass length and the diameter of the inner cell. In the present study, we changed the concentration of precipitant and that of protein manually. If these tasks were performed automatically using a computer-assisted system, the solubility measurements of protein crystals would be performed easily. Especially, when one uses polyethylene glycol (PEG) as a precipitant, such system will be useful since the time for exchanging of mother solution is clearly not short as compared with the time for exchanging of mother solution containing small ions. To maximize the rate of dialysis, in such case, the dialysis membrane with the largest weight cut off (MWCO) which will not cause excess loss of protein should be used.

The promotion of spontaneous nucleation of crystals of macromolecules generally requires excessive levels of supersaturation. On the other hand, it is clear that the largest and best crystals, in terms of diffraction and mechanical properties, are usually grown at the lowest level of supersaturation possible. If one knew the solubility curve of a particular protein for a specific precipitant, then this might present an important information to control the level of supersaturation. It should be noted that present our technique is useful not only for the precipitant dependent solubility measurement of protein crystals but also for the supersaturation control on protein crystallization, since the concentration of precipitant in crystallizing solution can be freely changed by exchanging the solution in the outer cell. We are now developing a crystallization device by which the level of supersaturation can be controlled dynamically based on the precipitant dependent solubility curve of protein crystals.

The authors wish to thank G. Sazaki for his advice on using the interferometer.

References

Ataka, M. & Tanaka, S. (1986). *Biopolymers* 25, 337-350.
Ataka, M. & Asai, J.(1988), *J.Crystal Growth*. 90, 86-93.
Komatsu, H., Miyashita, S. & Suzuki, Y. (1993). *Jpn. J. Appl. Phys*. 32, L1855-L1857.
Sazaki, G., Kurihara K., Nakada, T., Miyashita, S. & Komatsu, H. (1996). *J.Cryst.Growth*. 169, 355-360.
Sophianopoulos, A.J., Rhodes, C.K. , Holcomb, , D.N. & Van Holde, K.E.(1962). *J.Biol.Chem.* 237, 1107-1112.