Improvement of protein crystal quality by forced flow solution

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Flow experiments in growing protein crystals were conducted to clarify the influence of the solution flow on the crystal quality. Lysozyme crystals grown under various flow velocities were analyzed by using synchrotron radiation to assess the quality. As a result, the crystals grown under forced flow were of better quality than those grown in quiescent conditions.

Keywords: **protein crystal, quality, solution flow.**

1. Introduction

Single crystals are necessary to determine the three-dimensional structure of proteins by X-ray diffraction. Growing good crystals is the most difficult step in the process of structure determination. Growth condition fluctuations (vibration, temperature fluctuation, and solution flow) are known to influence the crystal quality. And they often lead to the occurrence of micro-crystals. Adhesion and incorporation of micro-crystals into larger crystals degrade the crystal quality due to introduction of disorders and strains. Furthermore, the protein concentration reduction induced by excess nucleation suppresses the growth. As a result, crystals can not reach suitable size for diffraction. For these reasons, crystallographers grow crystals in conditions as calm as possible, and the crystal growth method is designed to eliminate or minimize these undesirable effects.

The influence of solution flow has been investigated since solutal convection is essentially unavoidable as crystals grow. Convection affects growth kinetics, enhances impurity incorporation, morphological instability, inclusion formation, and nucleation (Wilcox, 1983). Hence, it has been widely recognized that high-quality crystals can be hardly obtained when solution flow exists.

However, Adachi *et al.* demonstrated a new method that decelerates the nucleation rate by stirring the solution (Adachi *et al.*, 2002). The gentle stirring accelerated the growth of protein crystals, prevented additional nucleation, and allowed large protein crystals to be obtained. This result suggested that carefully controlled flow may improve the crystal quality. However, the quality of crystals in terms of X-ray diffraction was not discussed. To reveal whether solution flow benefits the protein crystal quality or not is very important for protein structure determination. Therefore, the current study was carried out to examine the effect of solution flow on the protein crystal quality. We intended to estimate the crystal quality with respect to several properties measurable by X-ray synchrotron diffraction.

2. Experimental

2.1. Materials

Hen egg-white lysozyme (HEWL) used as the model protein in this study was purchased from Seikagaku Kogyo Corporation. The commercial lysozyme (Lot E99302) contained several impurities such as high molecular weight proteins and lysozyme dimers. The sample was purified to 99.99 % (w/w) to minimize the effects of impurities following a purification protocol reported previously (Thomas *et al.*, 1996). Purification was important since the increase of impurities in the crystals degrades their quality. Also, since forced flow is considered in this study, solution flow is thought to increase the transport rate of impurities in the crystal.

Solutions were prepared in 50 mM NaOAc buffer, pH = 4.5 and mixed with appropriate volumes of NaCl to obtain the desired final lysozyme (15 mg/ml) and NaCl (25 mg/ml) concentrations. Since the lysozyme solubility in 25 mg/ml NaCl and 50 mM NaOAc buffer at 10 °C is 5 mg/ml (Sazaki *et al.*, 1996), the supersaturation was calculated as SS = (15-5)/5 = 2 (200 %), using the relationship SS = (C-Ce)/Ce, where C is the protein concentration, and Ce is the protein solubility.

2.2. Flow system

Fig.1 shows the flow system, similar to that described by Vekilov & Rosenberger (1998), used in this work. The crystallization solution was circulated through a growth chamber by a peristaltic pump (ATTO Corporation; AC-2120). The chamber was placed in a temperature controlled incubator at 10 °C. The circulation loop consisted of silicon tubes and was kept at 24 °C to avoid additional nucleation. The solution was undersaturated at this temperature. The total volume of the solution was 20 ml. The average flow velocities were controlled by volumetric flow velocities from the 4 mm^2 cross-sectional area of the growth chamber.

We used seed crystals grown in a high supersaturation solution (SS=7.3). The seed crystals were placed in the flow cell with their *c*-axis parallel to the flow stream. Next, they were grown in a solution of 24 mg/ml Lysozyme, 25 mg/ml NaCl, 50 mM NaOAc buffer (pH 4.5) at 20 °C for 12 hours to fix them on glass substrates. In the actual flow experiment, the glass substrate was set upside down with the crystal stuck on the upper glass wall (Fig.1).

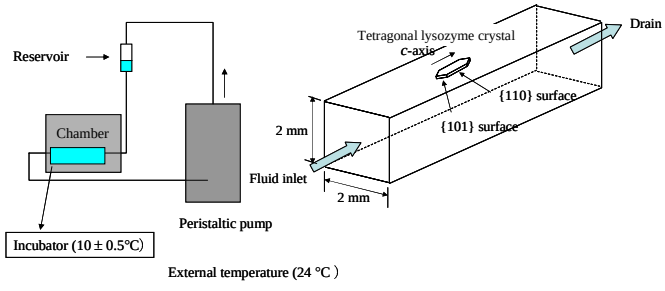

Figure 1
Schematic diagram of the flow system.

In studies of protein crystal growth under forced flow, much attention has been paid to protein denaturation (Durbin & Fether, 1986; Pusey *et al.*, 1988; Nyce & Rosenberger, 1991; Grant & Saville, 1995). The flocculation also changes the solution concentration and inhibits crystal growth. Therefore, we have to rely

on experimental conditions to eliminate the influence of denatured proteins.

We measured the lysozyme activity to check the protein denaturation. The lysis rate of Micrococcus lysodeikticus indicates the lysozyme activity. A micrococcus lysodeikticus (Sigma; Lot No .39H8615) suspension of 0.3 mg/ml in 0.1 M potassium phosphate buffer, pH 7, was prepared (Wang *et al.*, 1996). The crystallization solution circulated at various flow velocities and was then diluted with 0.1 M potassium phosphate buffer to the final protein concentration of 1 mg/ml. An aliquot of 20 µl diluted sample was added to 900 µl of micrococcus suspension. Then, the decrease in absorbance at 450 nm was recorded continuously with a spectrophotometer (Beckman; DU7500) for 1 min at 20 °C. The activity of lysozyme was determined from the initial slope. The difference in the activity indicated the occurrence of protein denaturation. Finally, it was decided to carry out experiments with 0 (quiescent), 100, 500 and 1000 µm/s flow because denaturation was not observed in these conditions. As expected, white flocculation did not occur at these flow velocities for at least two weeks.

2.3. X-ray diffraction data collection

When the crystals reached the size of about 100 × 60 × 400 µm, the growth was stopped and the crystals were transferred from the flow cell to a dish filled with an equilibrium solution at 24 °C. To remove the crystals from glass substrate, they were then gently blown off using a pipette. No physical damage was observed after removing the crystals. Afterwards, the crystals were mounted in glass capillaries with their long axis (*c*-axis) along the capillary. The X-ray beam was set perpendicular to the longer axis. Since highly directive X-ray beam was utilized, the illuminated crystal volume could be calculated as 0.1 mm (collimator size) × (crystal width) × (crystal thickness) mm. Because the size of the crystals was almost identical, the actual illuminated volume was effectively the same (see Table.1).

X-ray diffraction experiments were performed at room temperature at the BL-6A of the photon factory (PF), Tsukuba, Japan. Diffraction data sets were collected using a ADSC Quantum 4R CCD detector by the oscillation method with a wavelength of 0.978 Å. All X-ray experiments were performed under the same conditions. The crystal-to-detector distance was set to 100 mm in order to obtain adequate reflection separation. Exposure time per image was 15 seconds, and one image was collected with 1° oscillations. The quiescent- and flow-crystal data were collected by turns. The images were auto-indexed and integrated using the program DPS/MOSFLM/CCP4 (Rossman & van Beek, 1999) and then merged and scaled together with SCALA/CCP4 (Collaborative Computational project, 1994). Eight samples were analyzed in total. All investigated lysozyme crystals belonged to the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 79.1$ Å, $c = 38.0$ Å, $\alpha = \beta = \gamma = 90°$.

3. Result and discussion

The X-ray data statistics for crystals with and without forced flow are summarized in Table 1. The $\langle I\rangle/\langle\sigma(I)\rangle$ for quiescent- and flow-crystals were presented as a function of resolution in Fig.2. $\langle I\rangle/\langle\sigma(I)\rangle$ means the signal-to-noise (S/N) ratio of the diffracted intensity, where I is the diffraction intensity and $\langle\sigma(I)\rangle$ is the standard deviation obtained by merging symmetry-related reflections. Over the entire resolution range, the $\langle I\rangle/\langle\sigma(I)\rangle$ values of the flow-crystals were higher than those of quiescent-crystals although the crystals were of identical size. Also, a clear extension of the resolution limit to a higher value was noticed in flow-crystals. The improvements of flow-crystal data sets were due to higher average diffracting intensity and less intensity dispersion. This suggested the unexpectable result that the quality of all flow-crystals was better than that of quiescent-crystals.

In the range of 100 to 1000 µm/s, the differences in the overall $\langle I\rangle/\langle\sigma(I)\rangle$ values between crystals were smaller than those found between quiescent- and flow-crystals (Table. 1). An explanation for the dependence of crystal quality on flow velocity will be presented elsewhere.

The improvements in diffraction of flow-crystals were also shown in R_{merge}. R_{merge} is an overall measure of errors within a dataset. It compares the difference between symmetry-related reflections that should ideally be identical in intensity. The R_{merge} values are shown in Table 1. The values of flow-crystals were significantly smaller than those of quiescent-crystals. This result indicated that the flow-crystals have better agreement with the symmetry-related reflections.

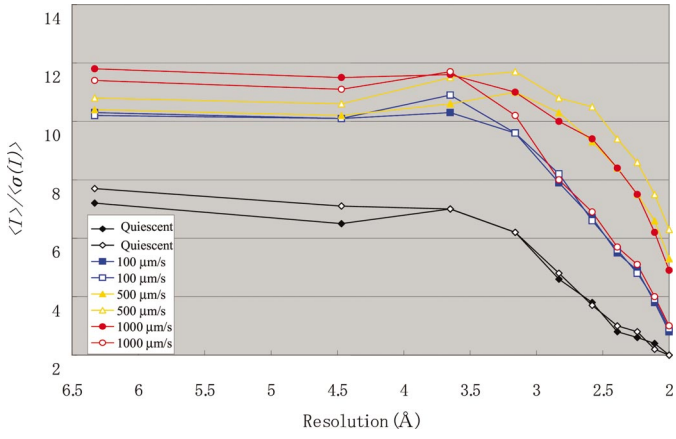

Figure 2

Comparison of diffraction data for quiescent- and flow-crystals showing the $\langle I\rangle/\langle\sigma(I)\rangle$ as a function of resolution. The flow-crystals clearly demonstrated improvement of diffraction properties and a higher resolution over the entire resolution range.

At present, the reason for this quality improvement is not clear. No differences in crystal shape, growth orientation, or cracks were observed between quiescent- and flow-crystals. However, it was found that step bunching occured by solution flow on the protein crystal surface by AFM observation (Rong *et al.*, 2003). Formation of step bunching has been well known as an effect of forced flow in solution crystal growth (Chernov *et al.*, 1986; Hasegawa, 1997). The step growth rate is likely to slow down with the formation of bunching. Slower crystal growth was reported to yield higher quality crystals (Yoshizaki *et al.*, 2001).

A possible improvement mechanism could be thought as follows. Once step bunching is formed by solution flow, the growth rate will decelerate owing to the increase of step-step interactions. During this slow growth, the number of growth unit around one kink site decreases with increasing in kink (step) density. More time will be available for a growth unit to orient and adapt to a minimum-energy attachment at the kink site before being interfered with subsequent growth unit. As a result, the growth units may be stabilized with an optimum configuration at the kink site, and the order of the molecule alignment rises.

Table 1

X-ray data collection statistics for flow- and quiescent-grown crystals of HWEL.

Sample No.	1	2	3	4	5	6	7	8
Flow velocity	Quiescent	Quiescent	100μm/s	100μm/s	500μm/s	500μm/s	1000μm/s	1000μm/s
Crystal dimensions (μm)	0.10 0.06 0.40	0.10 0.06 0.40	0.10 0.06 0.40	0.10 0.07 0.41	0.10 0.06 0.40	0.11 0.06 0.40	0.10 0.05 0.40	0.10 0.06 0.40
Space group	P43212	P43212	P43212	P43212	P43212	P43212	P43212	P43212
Cell parameters a=b (Å)	79.2	79.1	79.0	79.1	79.2	79.1	79.2	79.1
c (Å)	38.0	37.9	37.8	38.0	38.0	38.0	38.0	37.9
Oscillation angle(Å)	1	1	1	1	1	1	1	1
No. of images	180	180	180	180	180	180	180	180
Resolution range (Å)	10.0-2.0	10.0-2.0	10.0-2.0	10.0-2.0	10.0-2.0	10.0-2.0	10.0-2.0	10.0-2.0
Number of unique reflections	8329	8338	8275	8355	8368	8412	8326	8320
Overall Completeness (%)	97.3	97.9	97.4	97.6	98.4	97.6	97.2	97.6
Overall $<I>/<\sigma(I)>$	4.1	4.2	6.7	6.8	7.7	8.3	8.2	7.2
Overall *Rmerge* (%) †	12.5	12.4	8.7	8.5	6.0	6.4	6.4	8.3
Overall *B*-factor‡	19.2	17.4	16.6	17.7	17.5	17.2	16.7	16.7

† $R_{merg} = \sum|I_i - \langle I\rangle| / \sum I_i$ where I_i is the measured intensity of an individual reflection and $\langle I\rangle$ is the mean intensity of symmetry-related equivalent reflections.
‡ The values of the overall *B* factor were calculated from a Wilson plot (in the range 3.0 to 2.0 Å).

The morphological instability with respect to step bunching may be strongly influenced by the flow field. If the surface morphology affects protein crystal quality, the crystal quality varies with flow velocity. Therefore, the ultimate goal of this study is to find the optimum flow velocity for each protein.

To elucidate the quality improvement mechanisms, the dependence of the crystal growth rate and surface morphology change (step generation rate by 2D-surface nucleation, step density and bunching) with various flow velocities is currently studied. Moreover, in order to understand the growth mechanism from the transport process, we intend to investigate the changes of boundary layer thickness and concentration distribution around crystals using interferometry.

4. Conclusion

The effect of forced solution flow on the crystal quality of lysozyme crystal was investigated. Preliminary X-ray experiment results strongly suggested that the crystals grown under forced flow were of better quality. This demonstrated the possibility that controlled solution flow would improve protein crystal quality. Additional diffraction experiments, AFM observations, and interferometry experiments are in progress.

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