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Letter

Direct observation of bunching of elementary steps on protein crystals under forced flow conditions

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ABSTRACT

Bunching of elementary steps by solution flow is still not yet clarified for protein crystals. Hence, in this study, we observed elementary steps on crystal surfaces of model protein hen egg-white lysozyme (HEWL) under forced flow conditions, by our advanced optical microscopy. We found that in the case of a HEWL solution of 99.99% purity, forced flow changed bunched steps into elementary ones (debunching) on tetragonal HEWL crystals. In contrast, in the case of a HEWL solution of 98.5% purity, forced flow significantly induced bunching of elementary steps. These results indicate that in the case of HEWL crystals, the mass transfer of impurities is more significantly enhanced by forced solution flow than that of solute HEWL molecules. We also showed that forced flow induced the incorporation of microcrystals into a mother crystal and the subsequent formation of screw dislocations and spiral growth hillocks.

For a crystal growing in a solution, buoyancy convection plays a significantly important role in the mass transfer of solute and impurities from a bulk solution to a crystal surface [1]. However, since atomic force microscopy cannot be used under flow conditions, it has been very difficult to visualize individual elementary steps, although lateral growth of steps on a crystal surface is the most fundamental elementary process during the layer-by-layer growth of a crystal. Relatively recently, Sazaki et al. [2] succeeded in visualizing individual elementary steps on a protein crystal surface by laser confocal microscopy combined with differential interference contrast microscopy (LCM–DIM). Maruyama et al. [3] observed, by LCM–DIM, elementary steps on a surface of a tetragonal crystal of model protein hen egg-white lysozyme (HEWL) under forced flow conditions, and studied effects of solution flow on step velocities. They found that solution flow enhances mass transfer of both solute and impurities.

Bunching of steps is one of the major effects caused by solution flow during the growth of crystals. In the case of inorganic crystals, such as ammonium dihydrogen phosphate (ADP) and potassium dihydrogen phosphate (KDP), there are detailed studies in which the formation of bunching steps by solution flow was macroscopically observed by optical microscopy [4,5]. Such studies revealed that solution flow induces instability and then forms bunching steps on a downstream slope of a spiral growth hillock, where steps are moving in the same direction as the solution flows. In contrast, when the direction of solution flow is switched 180°, the bunching steps formed on the now up-stream slope disappear (debunching) and then bunching steps newly appear on the now down-stream steps. Such behavior is also modeled in detail [5].

However, as far as we know, there is no study on the formation of bunching steps by solution flow on a protein crystal, which plays a crucially important role in the structural analyses of protein molecules by X-ray and neutron diffraction. Hence, in this study, we tried to directly visualize bunching processes of elementary steps, for the first time, on a tetragonal crystal of model protein HEWL. We discussed the effects of solution flow on the mass transfer of solute and impurity proteins, from the viewpoint of the bunching and debunching of steps.

HEWL of 99.99% and 98.5% purities were purchased from Maruwa Food Industries, Inc. and Seikagaku Co., respectively (99.99% HEWL was discontinued). Other chemicals were of the analytical grade. Seed tetragonal HEWL crystals were grown at (20.0±0.1) °C from a solution containing 70 mg/mL HEWL of 98.5% purity, 25 mg/mL NaCl, and 50 mmol/L sodium acetate (pH 4.5).

An observation cell (Fig. 1(a)) was made of two glass plates of 0.17 mm thickness. The thickness of an HEWL solution between the two glass plates was 2.0 mm. The length and width of the cell were 20 mm and 5.0 mm, respectively. After the seed crystals

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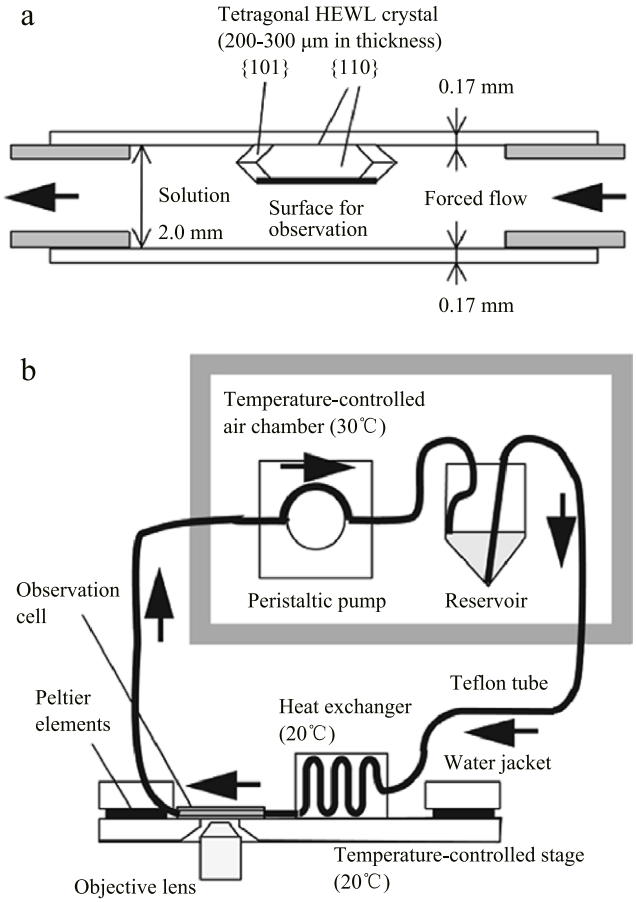

Fig. 1. Schematic illustrations of experimental setups: cross sectional views of (a) an observation cell and (b) a forced solution flow system.

had reached desirable size (0.2–0.3 mm), they were transferred to the observation cell filled with the solution of 98.5% HEWL. The observation cell was then placed upside-down for one day to attach the seed crystals on the ceiling of the cell.

Just before the observation, the cell was placed on a temperature-controlled stage in an upright way. Then, the solution inside the cell was replaced with a solution of 40 mg/mL HEWL of 99.99% purity using a flow system (Fig. 1(b)). The temperature of the cell was kept at (20.0±0.1) °C all through the experiment, using Peltier elements. In contrast, the temperature of a peristaltic pump and a reservoir in a temperature-controlled air chamber was kept at (30.0 ± 0.1) °C. An unsaturated HEWL solution in the reservoir (at 30.0 °C) was pumped through a Teflon tube. Then after the temperature of the HEWL solution was cooled to 20.0 °C by a heat exchanger, the supersaturated HEWL solution (at 20.0 °C) was pumped into the observation cell. The HEWL solution came out from the cell returned to the reservoir at 30.0 °C. Then micro HEWL crystals formed in the cell and tubes were dissolved in the reservoir. Flow rates were determined volumetrically.

The free surface (the {110} face marked by a bold line in Fig. 1(a)) of the seed crystals, which were placed parallel to the solution flow, were observed by LCM–DIM at a certain flow rate. Details of the LCM–DIM system were explained in our previous work [2]. During the growth of a crystal, a solute-depleted zone is generally formed in the vicinity of a crystal. However, since our seed crystal was attached to the ceiling of the cell (Fig. 1(a)), a lightweight solution in the solute-depleted zone could not go down by gravity. Hence, even under very slow forced flows, our observation system could minimize the effects of buoyancy convection during the observation.

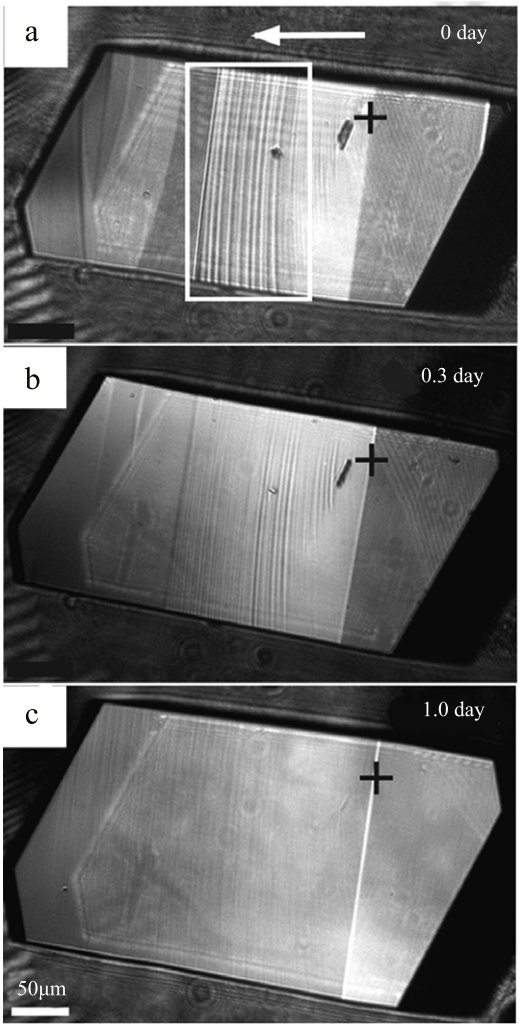

Fig. 2. A time course of a {110} face of a tetragonal HEWL crystal in a 99.99% purity HEWL solution at a flow rate of 220 μm/s. (a) 0 day. (b) 0.3 day. (c) 1.0 day. A cross mark and an arrow indicate the center of a spiral growth hillock and the direction of forced flow, respectively. A white rectangle in plot (a) shows bunched steps.

We first observed a {110} face of a tetragonal HEWL crystal on which bunched steps were formed by chance during the sample preparation. Fig. 2 shows a time course of the {110} face in a 99.99% HEWL solution at a flow rate of 220 μm/s. A cross mark indicates the center of a spiral growth hillock, which had a vicinal surface whose center exhibited the highest altitude. Since an arrow shows the direction of the forced flow, the right and left sides of the hillock corresponded to the up-stream and down-stream slopes of the hillock, respectively. At the beginning (Fig. 2(a)), the down-stream slope of the hillock exhibited bunched steps (marked by a white rectangle). Fig. 2 demonstrates that as time elapsed, the bunched steps disappeared. This result is opposite to those found on the spiral growth hillocks of ADP and KDP crystals [1]. We performed similar observations at the flow rates from 55 μm/s to 550 μm/s. However, at any flow rates, we could not find the formation of bunched steps by the forced flow on the downstream slope of the spiral growth hillock of the HEWL crystals. This result (Fig. 2) indicates that the coupling of bulk diffusion fields formed around adjacent steps on the HEWL crystal was weaker than in the cases of ADP and KDP crystals.

It is well known that a solution depletion zone, in which solute concentration becomes lower than in a bulk solution, is formed in the vicinity of a growing protein crystal. However, the growth rate