The growth of high-quality protein crystals is a prerequisite for the structure analysis of proteins by X-ray diffraction. However, dislocation-free perfect crystals such as silicon and diamond have been so far limited to only two kinds of protein crystals, such as glucose isomerase and ferritin crystals. It is expected that many other high-quality or dislocation-free protein crystals still exhibit some imperfection. The clarification of the cause of imperfection is essential for the improvement of crystallinity. Here, we explore twisting as a cause of the imperfection in high-quality protein crystals of hen egg-white lysozyme (HEWL) crystals with polymorphisms (different crystal forms) by digital X-ray topography with synchrotron radiation. The magnitude of the observed twisting is $10^{-6}$ to $10^{-5}$°/μm which is more than two orders smaller than $10^{-3}$ to $10^{-2}$°/μm in other twisted crystals owing to technique limitations with optical and electron microscopy. Twisting is clearly observed in small crystals or in the initial stage of crystal growth. It is uniformly relaxed with crystal growth and becomes smaller in larger crystals. Twisting is one of the main residual defects in high-quality crystals and determines the crystal perfection. Furthermore, it is presumed that the handedness of twisting can be ascribed to the anisotropic interaction of chiral protein molecules associated with asymmetric units in the crystal forms. This mechanism of twisting may correspond to the geometric frustration proposed as a primary mechanism of twisting in molecular crystals. Our finding provides insights for the understanding of growth mechanism and the growth control of high-quality crystals.

High-quality protein crystals are a prerequisite for the structure analysis of proteins by X-ray diffraction and the understanding of their intrinsic physical properties (1). Generally, it is difficult to grow high-quality protein crystals because of the complexity of the shape of constituent protein molecules and the intermolecular interaction, compared with those of simple inorganic and organic crystals (1). The assessment of crystal perfection with the characterization of crystal defects, such as dislocations, is important for the growth of high-quality protein crystals.

Many studies on perfection in protein crystals have been carried out based on rocking curve measurement, X-ray topography, and atomic force microscopy (2–7). Recently, it has been reported that dislocation-free perfect crystals such as silicon and diamond can be obtained even for protein crystals, which exhibit X-ray dynamical diffraction based on multiple scattering (8, 9). However, until now, such perfect crystals have been limited to only two kinds of protein crystals (glucose isomerase [GI] and ferritin). It is expected that many other high-quality or dislocation-free protein crystals still exhibit some imperfection. The clarification of the cause of imperfection is essential for the improvement of crystal quality and further understanding of the growth mechanism.

X-ray topography is one of the most powerful methods for the assessment of imperfection or crystal defects such as dislocation in single crystals (10, 11). Recent digital X-ray topography with a high-resolution charge-coupled device (CCD) camera can obtain local rocking curves with topographic images of crystal defects for single crystals, which are more useful for the evaluation of small distortions in the crystals (12, 13). Here, we report slight twisting as a cause of imperfection in high-quality or dislocation-free protein crystals of hen egg-white lysozyme (HEWL) crystals with polymorphisms (different crystal forms) by digital X-ray topography with synchrotron radiation.

Growth-induced twisting has long been known for various kinds of inorganic and organic crystals (14–18). For protein crystals, there are only a few studies on twisting in protein assemblies such as amyloid fibrils (19) and nanotubes (20) and ordered protein/silica structures produced by organisms (21), which are related to the cross-β structure, nonspecific electrostatic interaction, and Eshelby twist mechanism. Those protein assemblies have poor crystalline quality with strong twisting.

The magnitude of slight twisting in high-quality or dislocation-free protein crystals observed in this study is found to be the order of $10^{-6}$ to $10^{-5}$°/μm. Due to the technique

**Significance**

Growing high-quality protein crystals is a prerequisite for the structure analysis of proteins by X-ray diffraction. However, dislocation-free perfect protein crystals such as silicon and diamond are limited to two kinds of protein crystals. We wonder whether other high-quality or dislocation-free protein crystals still exhibit some imperfection. Here, we explore the existence of twisting as a cause of imperfection in high-quality protein crystals by X-ray topography with synchrotron radiation. The magnitude of twisting is quite small and cannot be detected by conventional techniques as optical and electron microscopy. The formation of twisting may be related to the geometric frustration mechanism proposed as a primary mechanism of twisting. This finding provides insights on high-quality protein crystals with the ubiquity of protein twisting.

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Results and Discussion

Fig. 1 shows typical digital X-ray topographic images (slice images) of tetragonal HEWL crystals, which were obtained by rotating the crystal to change the incident angle with 004 and 110 reflections. The white contrast with a band-like shape is partially observed in the crystal. The white band corresponds to the region of the high-intensity diffraction beam where Bragg diffraction occurs; this implies partial Bragg diffraction. It should be noted that the diffraction band is shifted in the direction perpendicular to or parallel to the rotation axis by rotating the crystal. From such behaviors, we can suppose that the handedness of twisting can be attributed to the anisotropic interaction of chiral protein molecules associated with asymmetric units in the crystal forms. These results may correspond to experimental evidence for the geometric frustration mechanism proposed with molecular dynamics simulation as a primary mechanism of twisting in molecular crystals (18, 22–24). This suggests that many other faceted protein crystals that look like perfect crystals also may include slight twisting. Our finding of slight twisting in high-quality protein crystals also provides insights for the understanding of growth mechanism and the growth control of high-quality crystals.

To examine the crystal quality, local rocking curves in the whole region of the crystal were analyzed. Fig. 2B–D shows the image obtained by digital X-ray topography, the profiles of the local rocking curves in some selected regions represented by circular areas of diameter of 96.75 μm (15 pixels), and the map of the full width at half maximum (FWHM) of the local rocking curves in the whole crystal. Generally, digital X-ray topographic images taken with a CCD camera correspond well with X-ray topographic images taken with X-ray films, giving rise to higher contrasts for local defects such as dislocations (26). The digital X-ray topographic image shows that the crystal is of relatively high quality and dislocation-free. The profiles of the local

Fig. 1. Experimental configurations and digital X-ray topographic images (slice images) of tetragonal HEWL crystal taken in (A), (B) 004 reflection and (C, D) 110 reflection. Schematic figures were prepared using the VESTA software (38). The slice images were obtained with the rotation of the crystal. The rotation axes in (A) and (C) are parallel to [110] and [001], respectively. The incident beam is almost vertical to (110) face.
rocking curves show a narrow single peak. In addition, the FWHM is almost uniform in the whole region of the crystal, as shown in Fig. 2D. The average value of FWHM is 0.0017°, which is comparable to those reported for high-quality protein crystals (2). Based on these images and map, the crystal is relatively high-quality, which implies that twisting occurs even in such high-quality crystals.

To evaluate the magnitude of twisting, the peak position (exact Bragg angle) of the local rocking curves was also analyzed. Fig. 2E shows the map of the peak position in the local rocking curves over the whole crystal. As shown in Fig. 2F, the peak position is plotted as a function of the location along a line corresponding to the direction of the twisting in Fig. 2E. Note that the location is measured relative to the midpoint on the line in the sample. The peak position changes linearly with the location except for the crystal edge. The value of the slope corresponds to the magnitude of twisting. Similar analysis on twisting has also been performed for the shift of the exact Bragg angle of nanocrystals obtained by electron diffraction (17) and ordered protein/silica structures produced by organisms obtained from crystal morphologies by optical and electron microscopy. Due to technique limitations, there are very few reports on the experimental evidence with high-quality crystals yet. In this mechanism, twisting is suggested to emerge from the preferred morphology at the molecular scale since molecules often assemble with some inevitable misfit.

Similar measurements and analyses were conducted for many of the different-sized high-quality tetragonal HEWL crystals grown in this study; the corresponding digital X-ray topographic images are shown in SI Appendix, Fig. S4. In the X-ray topographic images, the dislocations can be observed as the line shape contrasts. Some of the crystals are dislocation-free ones, and others exhibit a few dislocations. It should be noted that all crystals exhibit only left-handed twisting with or without dislocations. This means that the twisting observed in this work is not related to dislocation-associated Eshelby twist that is one of main causes of twisting (16–18, 21, 27–29). The investigated crystals also form no twin, which sometimes causes twisting (15).

It should be noted that the magnitude of the twisting is correlated with the size of the crystals, regardless of dislocations, as shown in Fig. 3A. Note that the crystal size means the length of the crystal along [110]. For comparison, correlation of the magnitude of twisting with the crystal size, which means the length of the crystal along [001], is shown in SI Appendix, Fig. S5. Fig. 3B shows the twist period, P, versus the crystal size, h, in logarithmic scale; the twist period corresponds to the length of a crystal needed for 2π rotation. The linear relationship fitted with a power law P \( \propto h^n \) gives \( n \approx 1.2 \) and 1.3 for crystals with and without dislocations, respectively. These behaviors are similar to those in previous reports (\( n = 0.6 \) to 1.3) (16). Moreover, it is considered that the dislocation has no effect to the twisting because the value \( n \) is almost the same. Both Fig. 3A and B show that the smaller the crystal size is, the larger is the magnitude of twisting, i.e., the magnitude of twisting is large in smaller crystals or those in the early stages of growth. Note that even the large twisting observed in this work is much smaller than those in previously reported twisted crystals (14–18). On the contrary, the magnitude of twisting is small in larger crystals or becomes small with the crystal growth. As shown in Fig. 2F, the magnitude of twisting is uniform over the whole crystal and is independent of the location even in larger crystals. These results suggest that the twisting in the crystals might uniformly relax with the crystal growth, and its magnitude decreases in large crystals.

As a primary mechanism of twisting in molecular crystals, geometric frustration has been recently proposed based on molecular dynamics simulations (18, 22–24), although there are very few reports on the experimental evidence with high-quality crystals yet. In this mechanism, twisting is suggested to emerge from the preferred morphology at the molecular scale since molecules often assemble with some inevitable misfit.
However, as twisting crystals get larger, they gradually untwist since the twisting is not preferred for long-range order. Such size-dependent behavior of twisting is one of the characteristics of the geometric frustration mechanism. In addition, it appears that the magnitude of twisting also depends on the characteristics, such as the shape, of constituent molecules in the crystals. Our results on size-dependent twisting (Fig. 3 A and B) may point to the geometric frustration mechanism, which still lacks extensive experimental evidence.

On the other hand, molecular nucleation mechanisms on protein crystallization have been recently investigated by liquid-cell transmission electron microscopy (TEM) (30) and cryo-TEM (30–32). Some TEM observations do not exhibit a highly ordered small structure, which is considered to be typical of classical nucleation process (30, 32). The crystal nucleation occurs because of amorphous solid aggregations or several ordered small domains emerging within one amorphous aggregate. The details of the amorphous phase or ordered small domains might depend on the kind of protein, such as HEWL (30), GI (31), and ferritin (32). Such amorphous phase can be also explained by the geometric frustration mechanism (23, 24).

Thus, the origin of the twisting in the crystals observed in this work might be related to the amorphous phase or ordered small domains in the nucleation process although our observation is limited in larger crystals after nucleation. Based on these results, we speculate that in protein crystals composed of chiral molecules, the molecular character associated with constituent proteins predominantly appears as twisting in smaller crystals or at the initial stage of crystal growth. Actually, the anisotropic interaction of chiral molecules during crystallization should give rise to twisting in the crystals. The molecular character-derived twisting can be relaxed or reduced with crystal growth, and the crystalline character becomes dominant for small twisting in larger crystals.

Furthermore, it is found that the magnitude of twisting is correlated with the FWHM exhibiting imperfection, as shown in Fig. 3C. The FWHM increases with increasing twisting magnitude in the crystals. This implies that the imperfection in high-quality protein crystals is primarily attributed to twisting. Such twisting might correspond to one of main residual defects in high-quality protein crystals. The growth of higher-quality crystals requires the suppression of twisting during crystal growth.

In this work, HEWL crystals with not only tetragonal form (P412121) but also other polymorphisms, such as orthorhombic (P212121), monoclinic (P21), and triclinic (P1) forms, were investigated. The direction of the shift of the peak position in the local rocking curve is shown with the crystallographic axis and direction of the rotation of the crystal for all forms in Fig. 4. The twisting for all crystal forms is clearly observed. The relationship between the crystal form and twisting axis always appears in all HEWL crystals as follows, tetragonal: c-axis, orthorhombic: c-axis, monoclinic: b-axis, and triclinic: a-axis. The three forms, i.e., tetragonal, orthorhombic, and monoclinic, have a screw axis, whereas the triclinic form has no screw axis. It is evident that twisting cannot be explained by the crystallographic screw axis. There is also no mirror plane in these polymorphisms. Thus, there is no direct correlation between symmetry of the crystal and presence of crystallographic symmetry elements.

As discussed earlier, the magnitude of twisting is large in smaller crystals or in the initial stages of crystal growth. In addition, the HEWL molecule, which is a typical protein, is a chiral molecule, as shown in SI Appendix, Fig. S6A. Thus, we infer that twisting in the crystals is attributed not to the crystallographic screw axis but to the anisotropic interaction of chiral molecules during crystallization.

Similar twisting is observed in other kinds of high-quality protein crystals, such as thaumatin crystal, although it has different twisting magnitudes, as shown in SI Appendix, Fig. S7. Thaumatin crystal is composed of chiral molecules, as shown in SI Appendix, Fig. S6B. Thus, it is suggested that the twisting associated with the anisotropic interaction of constituent molecules is a common phenomenon in protein crystals composed of chiral molecules.
On the other hand, only protein crystals, such as GI and ferritin, in which X-ray dynamical diffraction occurs have perfection degrees exceeding those of high-quality protein crystals such as HEWL and thaumatin (8, 9). They exhibit no clear twisting, as shown in SI Appendix, Fig. S8. This also supports that twisting is one of main residual defects in high-quality protein crystals. In addition, GI and ferritin molecules are made of 4 and 24 subunits, respectively (SI Appendix, Fig. S6 C and D). Both molecules have high symmetry with sphere-like shapes, in contrast to the low symmetry of HEWL and thaumatin molecules that have croissant-like shapes. In GI and ferritin crystals, it is speculated that there is an isotropic interaction between constituent molecules of high symmetrical shapes. The shape of constituent molecules is related to perfection and twisting in the crystals.

Finally, let us discuss the direction of twisting. The relationship between the direction of the peak position shift of the local rocking curve and the rotation axis for all crystal forms are shown in Fig. 4 and summarized in SI Appendix, Table S3. In this work, the measurements of the twisting have been carried out using 55 tetragonal, 7 orthorhombic, 8 monoclinic, and 6 triclinic HEWL crystals. All samples of tetragonal, orthorhombic, and triclinic HEWL crystals are left-handed twisting crystals. On the other hand, only monoclinic HEWL crystals are right-handed twisting crystals. Thus, the twisting sense (handedness) is the same for the same polymorph and crystal structure without any exception.

The handedness of twisting depends on the crystal form although all crystals are composed of identical HEWL chiral molecules. According to the molecular dynamics simulation of twisted molecular crystals such as benzil and 4-phenoxyniline, the handedness of the twisting is clearly determined to minimize the potential energy per molecule especially for small-sized crystals (23). The simulation shows that the twisted structure is more stable than the straight one. For protein crystals, HEWL molecules in tetragonal, orthorhombic, and triclinic forms can be arranged with a left rotation to obtain a stable structure. On the other hand, in the case of monoclinic HEWL crystals, it can be assumed that the HEWL molecules are stabilized by arrangement in a right rotation along the $b$-axis, which corresponds to the twisting axis. Here we suggest that the handedness of twisting may depend on the molecular interaction related to the asymmetric units in the crystal forms, as shown in SI Appendix, Fig. S9. Only monoclinic HEWL crystals have asymmetric units composed of two molecules, whereas asymmetric units in other forms comprise a single molecule. The discrepancy in the asymmetric units can be correlated with that in the handedness of twisting. Thus, it is presumed that the handedness of twisting is ascribed to the anisotropic interaction of chiral protein molecules associated with the asymmetric units in the crystal forms, although the mechanism is not clearly understood yet.

**Conclusion**

We have realized the characterization of growth-induced twisting in high-quality protein crystals such as HEWL crystals by digital X-ray topography. The characteristics of twisting can be ascribed to the shape of constituent molecules or asymmetric units composed of chiral proteins in the crystals. Thus, it is suggested that twisting is intrinsic or general phenomena in protein crystals of chiral or asymmetric molecules. In crystallization with proteins associated with very large twisting, such as common membrane proteins, it is difficult to relax the twisting during crystal growth. In this case, the crystals greatly twist so that it is difficult to obtain single crystals. Suppression of twisting is required for the growth of high-quality protein crystals.

**Materials and Methods**

**Crystal Growth.** HEWL was purchased from FUJIFILM Wako Pure Chemical Co. and Nacalai Tesque, Inc. and used without further purification. All other chemicals used for preparing solutions were of reagent grade. Large tetragonal HEWL crystals were grown using seed crystals chemically cross-linked by glutaraldehyde according to the procedure below (33). First, the seed crystals were grown from a crystallization solution containing 40 mg/mL HEWL, 0.5 M sodium chloride, and 0.1 M acetic acid-sodium acetate buffer (pH 4.5) at 21 °C using the hanging drop technique. For cross-linking, the seed crystals were immersed in 2.5 wt% glutaraldehyde with 0.5 M sodium chloride for 15 min at 23 °C. The cross-linked seed crystals were rinsed in 0.5 M sodium chloride solution to remove residuals on the crystal surface. Subsequently, the crystals were transferred to a handmade sample holder ($\phi = 30$ mm x $3$ mm) filled with a crystallization solution, which was filtered (pore size: 0.20 $\mu$m) to remove any particulate impurities or aggregates. To avoid heterogeneous nucleation, the holder containing the cross-linked seed crystal in the solution was annealed at 40 °C for 15 min. After annealing, the holder was kept at 23 °C for 2 wk to obtain millimeter-sized crystals. Similarly, orthorhombic (34), monoclinic (35), and triclinic HEWL crystals (36) were also obtained. The crystallization conditions and crystal structures are...
summarized in SI Appendix, Tables S1 and S2, respectively. Optical micrographs and corresponding schematic figures of the obtained HEWL crystals with four crystal forms are shown in SI Appendix, Fig. S1. The crystallization conditions for other protein crystals are described in the supplementary text.

**Digital X-ray Topography Measurements.** Synchrotron digital X-ray topography was performed at 23°C in BL14B and BL20B at the Photon Factory (PF) of the High-Energy Accelerator Research Organization (KEK). All experiments were conducted in the Laue geometry configuration, as shown in SI Appendix, Fig. S2. A monochromatic beam of 1.2 Å was selected by adjusting the double-crystal monochromator consisting of Si (111) crystals. An incident beam with a size of 3 × 5 mm² is sufficient to measure the entire crystal sample. Protein crystals sealed in the sample holder were mounted on the goniometer using wax after the growth solution in the sample holder was removed using a syringe. The crystal sample on a precision goniometer was rotated about an axis perpendicular to the incident beam with a high-resolution angular step (minimum angular step width: 0.19 arcsec [5.3 × 10⁻⁵]) around the exact Bragg angle. The digital X-ray topographs were recorded using an X-ray camera (Photonic Science X-RAY FDI 1.00:1, effective pixel size: 6.45 × 6.45 μm²). A series of digital X-ray topographs were obtained relative to the rotation of the sample crystal. The CCD camera outputs the sequential digital topographic images as 16-bit gray-scale TIFF files. To process the large amount of data in these images, we used the original computer software (26, 35, 37) to obtain mapping of the FWHM and peak position of the local rocking curves over the whole crystal. Further, the local rocking curve profiles were obtained from the reflected intensities in a selected circular area with a diameter of 96.75 μm (15 pixels) of the digital X-ray topographic image. Note that the size was determined by the effective size, which is sufficient to delineate the rocking curve using the reflected intensities. The averaged FWHM of the local rocking curves at three areas in the whole crystal were obtained. To evaluate the magnitude of twisting, the peak position of the local rocking curves was also analyzed, as shown in Fig. 2. The magnitudes of twisting were evaluated at three positions in the whole crystal, as shown in SI Appendix, Fig. S3. The averaged magnitudes of twisting are discussed in this work.

**Data Availability.** All study data are included in the article and/or SI Appendix.

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