

Epitaxial Growth of Trichosanthin Protein Crystals on Mica Surface

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ABSTRACT: We report the studies of epitaxial growth of trichosanthin (TCS) crystals on a mica substrate. TCS crystals grown on mica surface are found to be aligned well in four orientations, which was induced by the lattice of the mica substrate. The effect of potassium ions both at the mica surface and in solution was investigated by using different cation-modified surfaces and different precipitants. The results indicated that the binding of K^+ ions on the mica surface played a critical role in the epitaxial crystal growth of TCS. The high-resolution structures of crystals grown in different precipitants were solved by synchrotron radiation X-ray crystallography, and the K^+ binding sites in TCS crystals were identified. A strict lattice match between the TCS crystal and the mica substrate has been demonstrated based on the structural analysis of them. It reveals the mechanism of epitaxial nucleation and growth of TCS crystals. The experimental results provide new insights into the molecular mechanism of epitaxial growth and may open up the scope for the epitaxial crystal growth of proteins and other organic molecules.

Introduction

The growth of single, large crystals of macromolecules is the pivotal step for their structural determination by X-ray crystallography. However, to grow crystals of good quality is still a challenge and a major bottleneck for protein structural determination. Nucleation and crystal growth are the two fundamental steps of crystallization.¹ The ability to control these two steps is crucial for the control of macromolecular crystallization and has fundamental significance in developing efficient techniques for macromolecular crystallization.

Epitaxial growth has been widely used in preparation of inorganic crystals, but it has shown little success in macromolecular crystallization so far. This may be due to the difficulties in obtaining a strict geometrical match and the subtle balance of interactions between the macromolecules and the substrates (throughout the text, we use the word “substrate” to signify the surface on which crystals grow).

In the 1980s, McPherson and Shlichta¹ first used the approach of introducing an extraneous mineral as a heterogeneous surface to induce epitaxial nucleation and growth of proteins. Their work suggested that a direct lattice match was involved in the nucleation and growth of protein crystals on the mineral. Since then, all attempts to control the orientation of protein crystals on inorganic substrates were proven unsuccessful.^{2–9} As a successful example, the epitaxial growth of lysozyme crystals on fatty acid thin films was reported to be related to the carbon chain length of fatty acid molecules.⁹ A strict match of crystal lattice with the films could not be met owing to the fluctuation of periodicities of carbon chains adsorbed on the substrate. Still missing, for the mechanism of epitaxial growth of macromolecular crystals, is how the macromolecules interact with the substrates, and how the crystal lattices of the two very different materials match one another. These are known very well for the epitaxial crystal growth of inorganic materials.

On the other hand, there have been more reports in recent years of the epitaxial growth of peptide nanofilaments on such mineral substrates as mica and graphite.^{10–17} In these cases, the peptides are precisely assembled with the assistance of inorganic templates by electrostatic attraction¹⁴ or interfacial hydrophobicity/hydrophilicity¹⁵ between peptide and inorganic substrates. These features suggest that inorganic substrates might act as templates to induce large-scale order of supramolecular structure if one chooses inorganic substrate and experimental conditions properly.

In this study, we have demonstrated for the first time the epitaxial growth of the protein trichosanthin (TCS) on a hydrophilic mica surface with a precise match of molecular structures and have revealed the molecular mechanism of epitaxial growth of protein crystals in this case.

Experimental Section

Experimental Apparatus. The freshly cleaved mica has been used as the substrate in experiments. As mica surface is highly hydrophilic, the normal hanging drop method of protein crystallization does not work with the mica substrate. We have invented a new technique to use mica as the substrate of protein crystallization by sandwiching the droplet, which is shown in Figure S1 (Supporting Information).

Sample Preparations. TCS was extracted from the root tuber of the perennial plant Cucurbitaceae and was dissolved in deionized water for use. Mica (muscovite) was purchased from the Sichuan Yaan Mica Company. The crystallization trials were carried out at 291 K with a 24-well tissue-culture tray with a modified vapor-diffusion technique (Supporting Information). The TCS crystals were grown by equilibrating 3 μ L of TCS protein (40 mg mL⁻¹) mixed with 3 μ L of reservoir solution against a reservoir containing with 0.075 M sodium citrate (pH 5.4) and 14% (w/v) KCl over a period of 2–3 days.¹⁸

Cation-Modified Mica. The cation-modified mica surface was prepared by the method of S. Nishimura.¹⁹ Freshly cleaved mica was repeatedly rinsed with slightly acidic water (pH 5.8) until the wash water conductivity did not increase during the rinsing process. The H^+ -exchanged mica was immersed in 0.1 M NaCl, NiCl₂, LiCl, and KCl aqueous solutions for 12 h at ambient temperature to obtain Na^{+} -, Ni^{2+} -, Li^{+} -, and K^{+} -exchanged micas. After removal of the samples from the exchange solution, and without washing,

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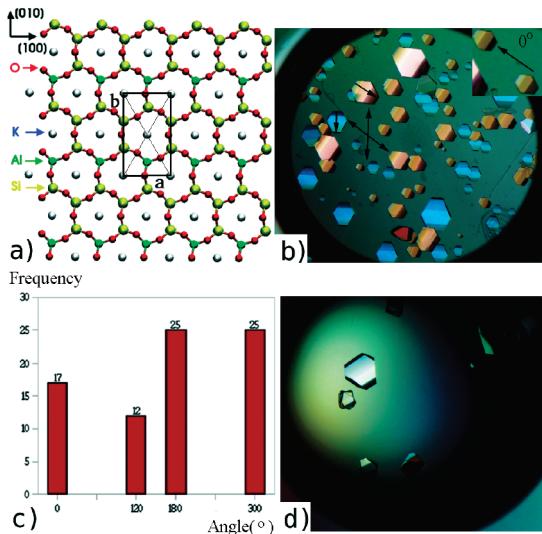


Figure 1. (a) Rectangular unit cell of the (001) plane of mica has side lengths of 5.19 Å and 9.00 Å (Supporting Information). (b) TCS crystals on mica surface; the arrows indicate four orientations of crystals. As all the crystals on mica surface have the same morphology with a hexagonal projection onto mica surface plane, the direction, which is perpendicular to two parallel edges of largest separation in the hexagon and points from long edge to short edge, has been defined to represent the orientation of crystal. For convenience, one orientation for a group of crystals has been set as the reference direction (0°) and the orientations of other crystals have been measured relative to the reference direction. Inset: two crystals related by twofold rotation; the arrow defines one of the four orientations. (c) Statistical histogram of the orientation distributions of crystals on mica. (d) TCS crystals grown on a normal glass coverslip by the hanging drop method.

these cation-exchanged micas were then calcined at 300 °C, followed by rinsing with water repeatedly until the water conductivity became equal to that of the fresh water. Then, the surfaces were blown dry with clean argon gas. These exchanged-calcined samples are referred to as Na, Ni, Li, and K-micas, respectively.

X-ray diffraction data for the structural determination of TCS crystals were all collected at SSRF BL17U1Macromolecular Crystallography Beamline.

Results and Discussion

Crystal Growth on a Mica Surface. Mica has a stacked sandwich structure with potassium ions located at the interlayer of the $\text{Al}_3\text{Si}_3\text{O}_{10}(\text{OH})_2$ hexagonal pattern.^{20–22} Upon cleavage, the K^+ sites appear on the (001) face, which has a rectangular lattice cell (Figure 1a). The TCS crystals grown on mica substrate, with uniform morphology, almost all aligned along four orientations around 0° , 120° , 180° , and 300° (Figure 1b,c), and adhered to the mica surface; while for the crystals grown on normal glass substrate at the same crystallization conditions (Figure 1d), they showed no orientations, and most crystals were actually found in bulk solution with much fewer crystals than on mica surface.

The crystal orientations on a mica surface have shown good consistency with the symmetry of the rectangular lattice of (001) mica surface (Figure 1a). It indicates the relationship between crystal orientations and the lattice structure of mica substrate. It also indicates that the mica surface helps to induce the epitaxial nucleation and the growth of TCS crystals on its surface, which has not been found before.

In order to investigate the effect of K^+ on the epitaxial growth, four kinds of mica surface, modified by cations Na^+ ,

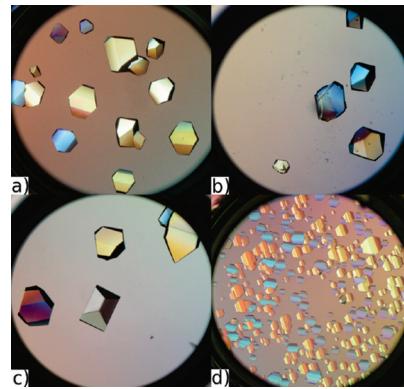


Figure 2. Effect of cation-modified mica surfaces on TCS crystal growth. The micrographs of TCS crystals grown on (a) Na^+ modified mica, (b) Ni^{2+} modified mica, (c) Li^+ modified mica, and K^+ modified mica.

Ni^{2+} , Li^+ , and K^+ , respectively, to replace the K^+ ions in the hexagonal cavities of mica surface,¹⁹ have also been used as the substrate in experiments. On the mica premodified with Na^+ , Ni^{2+} , and Li^+ , the number of crystals and their orientations were similar to that of crystals on normal glass, and no feature of epitaxial growth was observed (Figure 2a–c). By contrast, crystals on K-modified mica (Figure 2d) were aligned well not only along four orientations, but also with greatly increased number.

We investigated further the effect of K^+ in solution. When precipitant KCl was replaced by NaCl in the crystallization buffer for the crystal growth on a freshly cleaved mica surface, the aligned orientations of crystals no longer appeared and it just looked like crystal growth on normal glass (Supporting Information Figure S2a). When K-mica was used as the substrate in the experiment of precipitant NaCl, the situation remained similar to that on normal glass (Supporting Information, Figure S2b). It suggests that K^+ in solution is also critical for the epitaxial growth of TCS crystals. We think that, when K-mica is immersed in NaCl solution, K^+ ions in the hexagonal cavities of mica surface can be replaced by Na^+ , at least partly.^{23–25} These experimental results suggest that the array of K^+ ions on the mica surface is critical for the epitaxial crystal growth of TCS, and an interruption of the K^+ ion array by other ions may destroy the epitaxial crystal growth completely.

Lattice Match Between TCS Crystal and the Mica Surfaces. To investigate further the lattice match between TCS crystals and the mica surface, we performed structural studies of TCS crystals at SSRF Macromolecular Beamline BL17U1. We collected two sets of X-ray diffraction data for two crystals grown in KCl (K-TCS) and NaCl (Na-TCS) precipitant, respectively, and their structures were solved to a resolution of 1.03 Å and 1.18 Å, respectively (see Supporting Information). There is no difference between their crystal structures except for the bound metal ions (P1, P2, and P3 in Figure 3a). There are three metal ions in the K-TCS structure, and there is only one metal ion in Na-TCS structure. From the high-resolution structural analysis, three metal ions in K-TCS crystal can be reasonably assigned as K^+ and the metal ion in Na-TCS crystal assigned as Na^+ (Supporting Information, Figure S3), which was also identified by mass spectrometry (Supporting Information, Figure S4). The metal ions assigned in the structure have three or more coordination ligands and no regular coordination geometry.

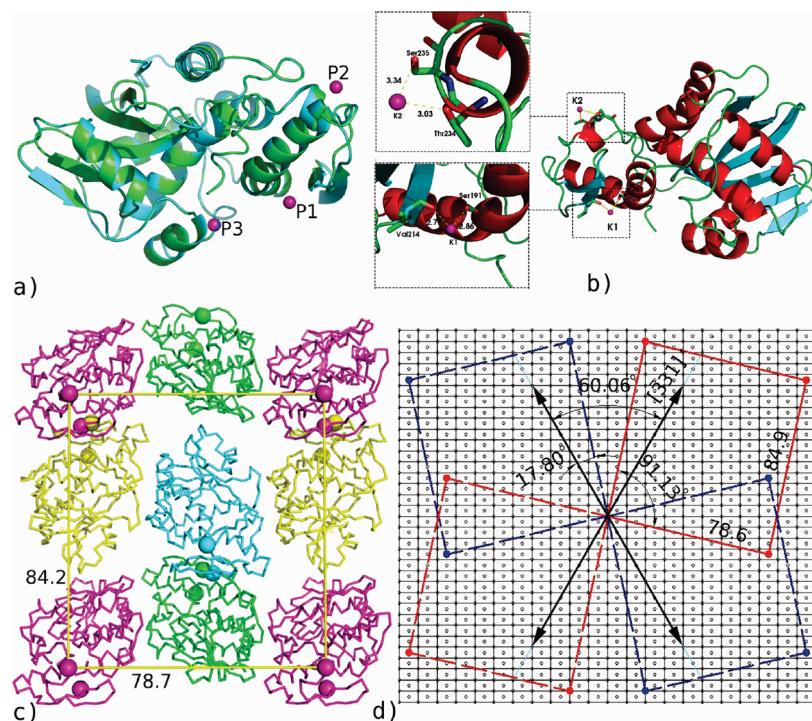


Figure 3. (a) Structures of K-TCS and Na-TCS crystals. The green represents K-TCS and the cyan represents Na-TCS. The pink spheres represent metal ions binding with K-TCS and blue (P3) represents the only metal ion binding with Na-TCS. (b) The structure of K-TCS crystal grown on mica. (c) K^+ binding sites on the $(110)/(1\bar{1}0)$ plane of TCS crystal form a rectangle lattice with a cell size of 84.2 \AA by 78.7 \AA , viewed along the crystallographic c axis. (d) The (110) plane of the TCS crystal superposes on the (001) face of mica with the K^+ binding sites fitting onto the K^+ array on mica surface. The best match is found with the lattice parameters of 84.9 \AA by 78.6 \AA on mica (red solid rectangle), in good agreement with TCS rectangular cell size. Three dashed rectangles are found by the rectangular symmetry of the solid rectangle. (Figure 3a–c was produced by PyMOL DeLano2002).²⁷

The distances between metal ions and the coordinated ligands varied from 2.7 to 3.5 Å. This result agrees well with the previous studies done by Harding.²⁶

The K^+ binding sites P1 and P2 (K1 and K2 in Figure 3b) are found on the surface of TCS, in which K1 locates right on the $(110)/(1\bar{1}0)$ cleavage plane and K2 is along the $(110)/(1\bar{1}0)$ plane but out of cleavage plane by about 10 Å. P3 is found inside TCS molecule. By measuring the X-ray diffraction of TCS crystal together with mica substrate, we confirm that it is the $(110)/(1\bar{1}0)$ plane of TCS crystal facing onto the (001) plane of mica (Supporting Information, Figure S5). The K^+ on $(110)/(1\bar{1}0)$ cleavage plane are arranged as a rectangular lattice (Figure 3c). This rectangular lattice can superpose quite well on the (001) face of mica and a perfect match has been found for two lattices on four orientations, which are related by the rectangular lattice symmetry of mica (Figure 3d). Along these four orientations of the TCS lattice, we can find the corresponding $[3\bar{3}1]$ directions along 0° (reference direction), 119.64° , 180° , and 299.64° (indicated by arrows in Figure 3d), which, we think, define the crystal orientations we observed.

We believe that we have shown that the epitaxial growth of TCS crystals on a freshly cleaved mica surface is guided by the array of K^+ on the mica surface, by interacting with the K^+ binding sites on the TCS molecules and providing an appropriate orientation and spacing. This explains clearly all the phenomena we observed in experiments.

Conclusion

We have demonstrated the epitaxial growth of TCS crystals on mica substrate. For the first time, the strict lattice match

between TCS crystals and the mica substrate has been established, based on the molecular structures of crystal and substrate, and the critical role of K^+ to induce the epitaxial nucleation and growth of TCS on mica surface has been elucidated. We believe that the mechanism being revealed here is heuristic and is applicable to more cases, e.g., to the proteins with other metal ion binding sites or with specific motifs. The insight into the molecular mechanism makes it possible to design the proper systems for the epitaxial crystal growth of proteins and other organic molecules on the natural or artificial ordered substrates, which, besides serving as a new method for protein crystallization, can also be envisioned as a unique approach to fabricate specific nanostructures.

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Supporting Information Available: Additional figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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