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Crystal growth of macromolecular crystals: correlation between crystal symmetry and growth mechanisms

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Abstract

In situ atomic force microscopy was utilized to study the relation between crystal structure, surface morphology, and growth mechanisms of faces of macromolecular crystals that have screw axes perpendicular to them. It was found that the {0 0 1} faces of orthorhombic catalase, trigonal trypsin, and tetragonal Bence-Jones protein (BJP) crystals grow by successive deposition of n alternating, symmetry-related layers with a thickness of $d_{0\,0\,n} = \frac{1}{n}|c|$. These layers are associated with two-, three- and four-fold screw-axes in the crystal structure of catalase ($n = 2$), trypsin ($n = 3$) and BJP ($n = 4$) crystals, respectively. Implications for growth rates and three-dimensional morphological development are discussed.

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1. Introduction

Correlation between crystal structure and morphology has been the subject of intensive studies for more than a century. For example, the Bravais Friedel Donnay Harker (BFDH) criterion [1,2] couples the interplanar distance $d_{h\,k\,l}$ of a face ($h\,k\,l$) to its growth rate R. Based on geometrical considerations and growth rates, it was demonstrated that the larger the interplanar distance $d_{h\,k\,l}$, the lower the growth rate of the face ($h\,k\,l$). This results in a higher relative statistical frequency of occurrence of ($h\,k\,l$) as well as a higher relative size of the ($h\,k\,l$) face occurring on a crystal, i.e. a larger morphological importance (MI) of the ($h\,k\,l$) face [1,3]. Within the BFDH criterion, corrections for $d_{h\,k\,l}$ were made for crystals containing non-primitive cells, screw axes and glide planes. While the BFDH model gives a satisfactory description of crystal morphologies in some cases, it often fails simply because it does not take into account the structure of the crystal and the bond strengths. Since BFDH, morphology prediction methods have evolved, largely on the basis of the periodic bond chain (PBC) theory developed by Hartman and Perdok [4]. PBC analysis requires information on the thickness of the growth layer, which is determined by the underlying crystal symmetry. Generally, macromolecular crystals contain multiple asymmetric units per unit cell [5], and the thickness of the

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growth layer depends on the presence of symmetry elements such a screw axes and glide planes. Thus, growth layers on the crystalline surface having heights equal either to the unit cell parameter [6] or to a fraction [7] were observed.

Here we present the results of in situ AFM studies of correlations between surface morphologies and crystal symmetry during the growth of {0 0 1} faces of catalase, trypsin and Bence-Jones protein (BJP) crystals having, respectively, two-, three-, or four-fold screw axes perpendicular to these faces.

2. Experimental procedure

We examined surface morphologies and growth mechanisms for developing crystals of catalase, trypsin and BJP using in situ AFM with a Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA, USA), operated in both contact and tapping modes under controlled supersaturation conditions, at 25°C, according to established methods [7,8]. Crystallization conditions for each protein as well as relevant molecular and crystallization properties are presented in Table 1.

3. Results

Growth of the {0 0 1} faces of catalase crystals proceeds exclusively by two-dimensional nucleation and layer-by-layer step advancement. The step height of the nuclei is equal to 11.5 ± 0.2 nm, which corresponds to half of the unit cell dimension $d_{0\,0\,2}$ along c. Each successive layer derived from a 2D island is related by a 180°, 2-fold rotation to the preceding layer (Fig. 1a). The anisotropic shape of the nuclei is due to the strong anisotropy in the step advancement rates in different crystallographic directions [7].

The formation of two alternating growth layers on the {0 0 1} face of catalase crystals is caused by the presence of the 2_1 screw axis in the structure perpendicular to this face. This screw axis divides the unit cell in two symmetrically equivalent layers rotated by 180°, which indeed should lead to the observed equivalent but rotated half-layers. Since there are four molecules in the unit cell, there are potentially three ways to distribute them into two equivalent half-layers. By analyzing the strength of interactions between the four molecules the most likely distribution was determined [7]. The anisotropy within a $d_{0\,0\,2}$ layer itself can be explained by the fact that the symmetry axes of the catalase tetramers (that posses 222 symmetry) do not coincide with the crystallographic directions, thus molecules forming step edges moving in opposite crystallographic directions expose different parts of their surfaces. This can result in different distributions of charges, hydrogen bond donors and acceptors, and hydrophobic clusters on the step edges as well as differential screening by ions. This alone could explain the different rates of molecule incorporation into $d_{0\,0\,2}$ step edges advancing in opposite directions [7].

Imaging of the hexagonal {0 0 1} face of trigonal trypsin crystals showed intense 2D nucleation,

Table 1
Crystal parameters and crystallization conditions

Protein	M_w (kD)	Space group	a (Å)	b (Å)	c (Å)	Z[a]	Crystallization conditions
Beef liver catalase (tetramer)	250	$P2_12_12_1$ [7]	87.8	140.8	233.4	4	Batch; 0.025 M magnesium formate, pH 7
Bovine pancreas trypsin	23	$P3_121$ [8,9]	54.82	54.82	109.8	6	Vapor diffusion; 2 M ammonium sulfate, 0.1 M tris, pH 8.5
Human BJP (dimer)	46	$P4_322$	64.2	64.2	179.6	8	Vapor diffusion; 1.4 M ammonium sulfate, pH 7.5–8.5

[a] Z = number of molecules in the unit cell.

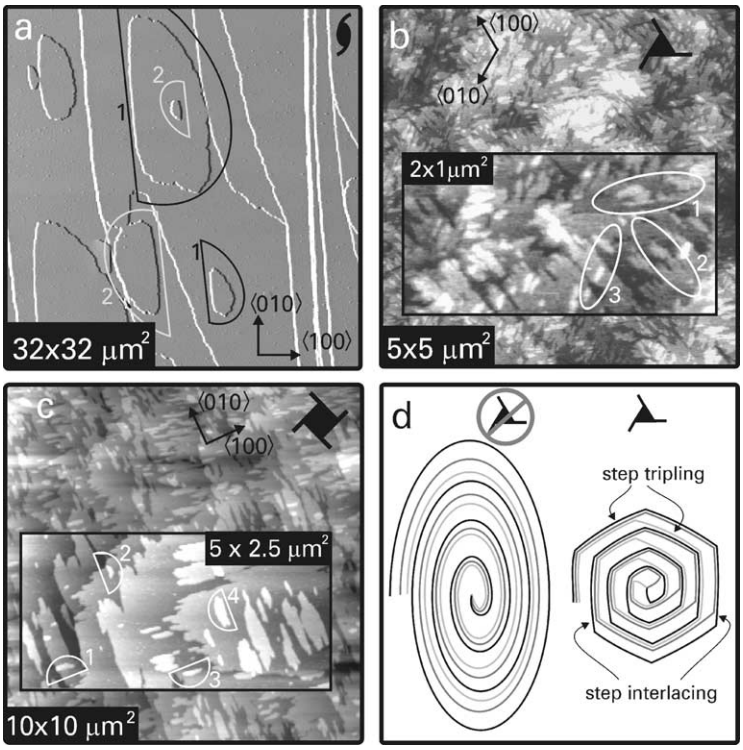

Fig. 1. (a–c) In situ AFM images of 2D nucleation on crystal faces perpendicular to screw axes (indicated in top right of images). Symmetry-related 2D nuclei are indicated by numbered (half) ellipse. The (0 0 1) faces of orthorhombic catalase (a), trigonal trypsin (b) and tetragonal BJP (c), which are perpendicular to 2_1, 3_1 and 4_3 screw axes, respectively. (d) A triple growth spiral in the case of crystal symmetry both without (left), and with (right) a 3_1 screw axis parallel to the screw dislocation. In the latter case, steps of subsequent layers are constraining each other, which leads to step tripling and interlacing. Note that in this case, with a 2:1 step velocity anisotropy ratio, the screw axis causes a 50% reduction in spiral size with respect to the unconstrained case.

while no growth spirals were observed [8]. The shape of the nuclei resembles an elongated ellipse. The elongation is in the direction parallel to the crystal edges, i.e. along $\langle 1\,0\,0 \rangle$ directions, with an average width/length anisotropy ratio of 1:4 (Fig. 1b). The shape of the nuclei forming each successive growth layer of thickness 3.2 ± 0.5 nm is related by a counterclockwise rotation of 120° with respect to the shape of the initiating nuclei of the preceding layer. This observed 3-fold symmetric growth behavior of the trigonal {0 0 1} face can be explained by the presence of the 3_1 screw axis parallel to c in the $P3_121$ crystal structure (note that, in case of a 3_2 axis, the new layers would have a *clockwise* rotation). The six molecules in the unit cell with a height of $c = 10.9$ nm can, in accordance with the space group symmetry, be sorted into three groups consisting of two molecules each, that are mutually related by a 3-fold screw axis. This results in three triad-related growth layers within a unit cell, each with a height of $d_{0\,0\,3} = \frac{1}{3}|c| = 3.6$ nm, which corresponds well to the experimentally observed growth steps of height 3.2 ± 0.5 nm.

Similar to the case for catalase crystals, the trigonal trypsin unit cell permits multiple divisions of the six molecules into three equivalent layers. On the basis of the bond strengths between the molecules, again the most probably distribution was selected [8]. Also, the shape of the 2D nuclei was predicted on the basis of the bond structure. This predicted shape correlates well with those experimentally observed [8].

In case of growth of the {0 0 1} face of tetragonal BJP crystals, symmetry-related layers produced exclusively by 2D nucleation were again

encountered. The shape of newly formed 2D nuclei resembled half ellipses, somewhat similar to the ones described above for crystallization of catalase. However, in case of the BJP crystallization, four subsequent growth layers were distinguished. These were related by a rotation of 90° clockwise for each subsequent layer (see Fig. 1c). The step height of the nuclei was 4.6 ± 0.2 nm. This corresponds well to $d_{0\,0\,4} = 4.49$ nm. These observations indicate that the observed symmetry-related layers are caused by the presence of the 4_3 screw axis (if there were a 4_1 screw axis, successive layers would exhibit a 90° *counterclockwise* rotation). The anisotropy within one growth layer further indicates that there are no symmetry elements that can mask the presence of the 4_3 axis, such as rotation axes, present within that layer.

4. General relation between screw axes and surface morphology

Generally, crystal faces in direction $(h\,k\,l)$ are expected to grow with layers of thickness $d_{h\,k\,l}$. The BFDH criterion [1] contains exemptions for structures with centered cells, glide planes and screw axes, which all lead to reduced growth layer thickness. Orthorhombic catalase, trigonal trypsin and tetragonal BJP all follow the exemption rule which implies that the existence of a screw axis in a crystal structure leads to the formation of reduced growth layers d_{nhnknl} on the face $(h\,k\,l)$ perpendicular to that axis. In the case of screw axes perpendicular to $\{0\,0\,1\}$, growth layers have a thickness of $d_{0\,0\,n}$. From symmetry considerations, the unit cell should be divided into two equivalent layers for 2_1, 4_2 and 6_3 screw axes ($n = 2$), three equivalent layers for 3_1, 3_2, 6_2 and 6_4 ($n = 3$) four layers for 4_1 and 4_3 ($n = 4$) and six layers for 6_1 and 6_5 ($n = 6$).

However, in order for the n-fold screw symmetry to influence the surface *morphology*, besides the growth layer thickness, there is another condition that must be fulfilled: The individual $d_{0\,0\,n}$ layers should be anisotropic in morphology and hence in structure. In other words, apart from the screw axes, there must not be additional symmetry elements that make the partial layers themselves symmetric. An example where this anisotropy condition is relaxed and screw axis-related symmetry is not visible, is for the growth of the $\{1\,1\,0\}$ face of tetragonal lysozyme. This crystal has a $P4_32_12$ symmetry, with 2_1 screw axes as well as 2-fold rotation axes perpendicular to the $\{1\,1\,0\}$ faces. Because of the 2_1 axes, a surface pattern similar to that of catalase could be expected. However, due to the 2-fold rotation axes, there is a 2-fold rotation symmetry in the individual growth layers present, so the effect of the 2_1 axes is not evident. Indeed, with regard surface morphology the successive layers have exactly the same, 2-fold symmetry [10,11].

In cases where both conditions (i.e. presence of screw axes and absence of further symmetry) are fulfilled, successive layers are rotated by 60°, 90°, 120° or 180°, existing layers will hinder growth of newer layers because of the anisotropy in step velocity. If a 2D nucleus grows atop another nucleus originating in the previous layer, steps in the fastest direction of the superior nucleus will eventually overtake slower moving steps of the inferior nucleus in the same direction, forming a double step. Further growth of the upper nucleus is blocked until the slower steps of the lower nucleus advance. In case of growth by 2D nucleation this effect is rather limited because of random multiple 2D nucleation. However, in the case of a growth spiral originating from a screw dislocation, the origin of each new growth layer is pinned to exactly the same point (i.e. the dislocation outcrop), and therefore successive symmetry-related layers demonstrating rotated step velocity anisotropy can be significantly restrained, as is shown in Fig. 1d. For crystal faces with relatively low anisotropy it is still possible for dislocation spirals to grow, and they will develop growth patterns with multiple (e.g. double for 2_1 or triple for 3_1) growth steps. In growth directions where small and large step velocities of subsequent layers change, the pairing of steps will alternate. This leads to the formation of "zig-zag" patterns known as 'step interlacing' [12,13] (see Fig. 1d). In the case of macromolecular crystals, interlacing at a spiral was observed for the $\{0\,0\,1\}$ face of a 50S ribosome crystal with space group $C222_1$ (which has a 2_1 screw axis perpendicular to the

{0 0 1} face) [14]. The greater anisotropy of the growth layers, the more severe the steps restrain one another. Eventually, this can lead to 'self-inhibition' of growth spirals, as was shown for the case of *Pnma* barite {0 0 1}, having screw axes parallel to *c* [15]. In this case, the growth rate of the spirals was so reduced (because of step hindrance) that 2D nucleation alone determined the growth rate of the face.

Growth of the three macromolecular crystals we investigated proceeded strictly by the 2D nucleation mechanism; no dislocation spirals were ever observed over a wide supersaturation range. In the case of catalase crystals, this can probably be explained by their high elasticity that appears to prevent formation of dislocations [7]. Other crystal forms of both trypsin and BJP, however, showed extensive dislocation spiral growth [8]. The absence of dislocation spirals on the {0 0 1} faces investigated here, together with their high degree of step velocity anisotropy, suggest that self-inhibition of growth spirals may have taken place in these cases.

Crystal growth at a low supersaturation where the barrier for 2D nucleation is excessive, generally proceeds by the spiral growth mechanism [16]. The self-inhibition mechanism produces an obstacle to spiral growth at both high *and* low supersaturation. Hence, growth of crystal faces constrained by spiral self-inhibition is influenced in two ways: (i) At low supersaturations, where only spiral growth can take place, the growth rate R of the face is severely reduced. (ii) The degree of supersaturation at which 2D nucleation begins to dominate over spiral growth is lowered. The first, *i*, results in an increase of the morphological importance, MI, of these faces: they will more strongly influence the crystal habit then they would do without this effect. Because many macromolecular crystals contain screw axes, this may be of significant importance for morphology prediction. To determine the impact of screw axis-induced effects, Monte Carlo methods that can simulate screw dislocations in the presence of screw axes should be developed. Alternatively, statistical analyses of the MI of the faces of a large number of macromolecular crystals could be undertaken.

5. Conclusions

A direct relation between the presence of screw axes in a crystal structure and the surface morphology of crystal faces perpendicular to it was found for the cases of catalase, trypsin and BJP protein. In all cases, the crystal faces showed anisotropic 2D islands in subsequent layers, which were related to each other by screw axis symmetry. The occurrence of this phenomenon was generalized to all possible screw axes, and was also restricted to cases without further symmetry elements.

As a consequence of step hindering of steps of subsequent layers, growth spirals may self-inhibit themselves, which eventually leads to lower growth rates of the faces involved at low supersaturation and a widening of the supersaturation range where 2D nucleation dominates over spiral growth.

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