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The influence of precipitant concentration on macromolecular crystal growth mechanisms

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Abstract

Atomic force microscopy was applied to investigate the influence of protein and precipitant (sodium-potassium tartrate) concentration on thaumatin crystal growth mechanisms. At constant protein concentration, a decrease of salt concentration from 0.8 to 0.085 M caused a transition of the crystal growth mechanism from two-dimensional nucleation to dislocation growth. At different, fixed concentrations of salt, the protein concentration, which does not induce multiple crystal nucleation, was increased from 8 to 60 mg/ml with corresponding increases in the tangential velocity of growth steps from 5 to 17.5 nm/s. Results from these experiments suggest that a highly concentrated protein solution, as might be found in a protein rich phase, may not induce crystal nucleation, but can promote crystal growth if screw dislocations are present in the crystal.

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1. Introduction

Unless seeding is employed, macromolecular crystals are always grown at very high levels of supersaturation necessary for nucleation, but supersaturation ranges non-ideal for growth. Low or moderate protein concentrations with high precipitant concentrations are generally employed. With high protein concentrations, multiple nucleation is a common experience with the consequence that few, if any, crystals grow large. At low protein concentration nucleation is reduced, but again crystals may fail to grow large because too little protein is available. This is primarily due to the fact that at high precipitant concentrations, crystals grow predominantly by the mechanism of two-dimensional nucleation, a mechanism that is particularly sensitive to, and limited by protein concentration. This has been demonstrated for a number of macromolecular crystals by AFM [1–9].

We have further studied the growth of thaumatin crystals [10,11] under a variety of protein concentrations and salt concentrations using AFM. This investigation, we believe, contributes to a better understanding of the considerations involved in choosing optimal crystallization conditions for macromolecules.

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2. Experimental procedures

2.1. Material

Thaumatin is a protein ($M_r = 22\,000$ Da) from the arils of the African shrub *Thaumatoccus daniellii,* and was purchased from Sigma Biochemical Co. The structure of the tetragonal crystals studied here ($P4_12_12$, $a = b = 5.86$ nm, $c = 15.18$ nm) has been determined by X-ray diffraction [12]. Conditions for its crystallization, and other properties of its crystal growth have been presented elsewhere [2]. Approximately 50–60 thaumatin crystals were investigated during the course of this study. The crystals were tetragonal bipyramids with {1 0 1} faces and an edge length of 0.3–0.5 mm.

2.2. Methods

Crystal seeds were nucleated and grown by a batch method which consisted of mixing aliquots of 20 mg/ml protein in water with equal amounts of 1.6 M sodium-potassium tartarate (0.1 M ADA, pH = 6.5). A crystal seed was then mounted beneath carbon fibers on a cover glass, submerged in a 20 μl droplet of experimental solution, and the glass slip was transferred to the fluid cell of an atomic force microscope (Nanoscope E, Digital Instruments, Santa Barbara, CA). Images were collected at 29°C in contact mode. To avoid crystal cracking due to the difference in salt concentration between the inside of the crystal and the surrounding solution, whenever crystal seeds were moved into different concentrated salt solutions, the concentration of the precipitant in the seeded solution was slowly changed. Generally, thaumatin crystals crack when moved from a solution of high to low salt concentration. As salt ions begin to diffuse from the crystal into the solution, the salt concentration gradient creates tensile stress in crystal outer layers. This is because the lattice parameters attempt there to decrease compared with the parameters of the lattice in the interior of the crystal.

3. Results and discussion

3.1. Protein equilibrium concentration

In our previous studies [10], we assumed that the equilibrium concentration of thaumatin was 2 mg/ml in the presence of 0.8 M sodium-potassium tartarate at pH = 6.5, as no growth was observed at lower protein concentrations. Further studies, however, demonstrated that there is no detachment of protein molecules from the crystal growth step edges even with precipitant concentrations as low as 0.085 M in the complete absence of protein. Only after several days are any traces of etching on crystal surfaces noticeable. Observations of the growth step structure in solutions of low precipitant and protein concentration imply that there is no exchange of protein molecules between crystal and solution [13]. It means that the interaction energy between molecules in the crystal is high and that thermal energy is insufficient to produce detachment of molecules from the crystal, even at low salt concentrations. As a consequence, we studied the kinetics of the growth step velocity as a function of protein concentration.

3.2. Growth mechanisms

In protein solutions of high salt concentration used for growing thaumatin crystals, 0.8 M, growth proceeds by two-dimensional nucleation even at low, 3 mg/ml, protein concentrations. There is a strong kinetic anisotropy in the step advancement for thaumatin crystallization [10,11]. On the (1 0 1) crystal face, the rate of growth step advancement in the direction $[\bar{1}\,0\,1]$ is approximately three times that of the opposite direction $[1\,0\,\bar{1}]$. At a protein concentration of 3 mg/ml, the growth step does not move in the direction $[1\,0\,\bar{1}]$ at all, but has a velocity in the opposite direction of 0.5 nm/s. Under these growth conditions, points of emergence of screw dislocations on the crystal surface can be observed. These screw dislocations do not form hillocks, but create spirals with no more than one turn, Fig. 1(a). Most of the crystal face, however, becomes covered with growth steps formed by two-dimensional nucleation. Increase in

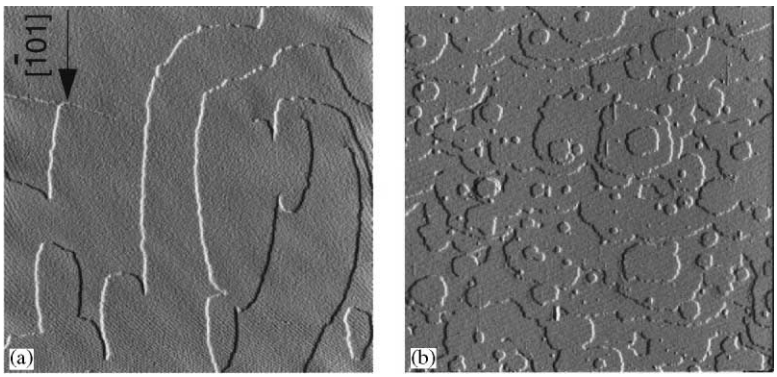

Fig. 1. Face (1 0 1) of growing thaumatin crystal in solutions with precipitant concentration 0.8 M and protein concentrations (a) 3 mg/ml, scan size $7 \times 7\,\mu m^2$ and (b) 8 mg/ml, scan size $10 \times 10\,\mu m^2$.

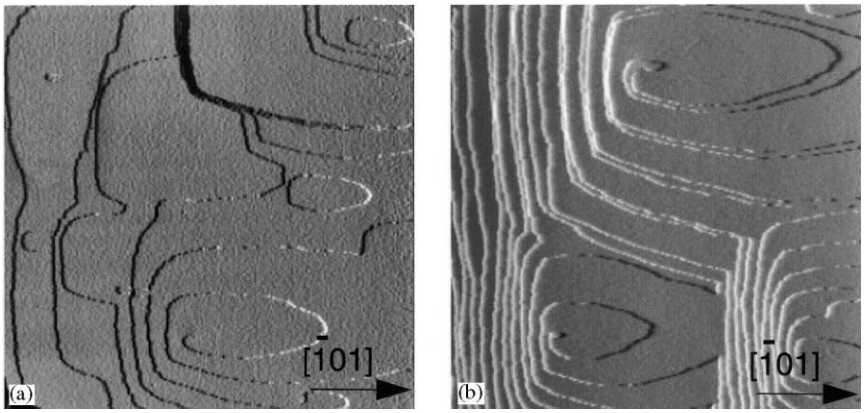

Fig. 2. Surface of growing thaumatin crystals in solutions with concentrations of (a) 0.16 M salt and 25 mg/ml protein and (b) 0.085 M salt and 30 mg/ml protein. Scan size $15 \times 15\,\mu m^2$.

protein concentration increases the rate of two-dimensional nucleation and, eventually, only growing islands are apparent on the crystal face, Fig. 1(b). Gradual decrease of the precipitant concentration at low protein concentrations makes the dislocation growth mechanism more pronounced. Finally, at a salt concentration of about 0.1 M, well-developed dislocation hillocks cover the crystal face at all protein concentrations, Fig. 2(a). No two-dimensional islands are observed at all, even at protein concentrations up to 60 mg/ml, Fig. 2(b).

3.3. Growth step kinetics

In solutions of high salt concentration, a relatively small increase in protein concentration, up to 8 mg/ml, dramatically increases the tangential velocity of growth steps to 8 nm/sec for the direction [$\bar{1}$ 0 1], Fig. 3. Growth steps of nucleated two-dimensional islands merged so rapidly that we were unable to measure the growth step tangential velocities at higher protein concentrations. A vast number of microcrystals also nucleate in solution when both salt and protein concentrations are high.

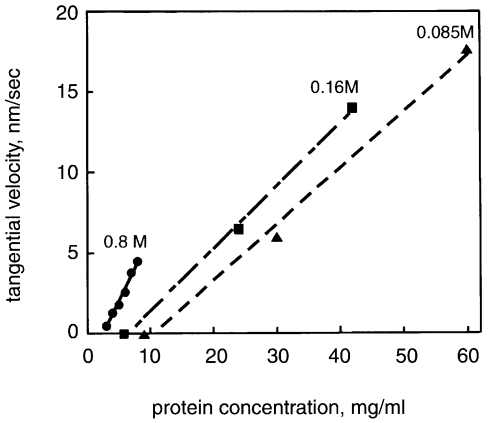

Fig. 3. Dependencies of tangential velocities of growth steps in the direction [$\bar{1}$ 0 1] at precipitant concentrations: A: 0.8 M, B: 0.16 M and C: 0.085 M.

A decrease in salt concentration caused a corresponding decrease in the kinetic coefficient of the growth steps from 10^{-6} m/s at 0.8 M salt concentration to 10^{-7} m/s at 0.085 M, but permitted an increase in the maximum concentration of protein in solution. As a consequence, the maximum tangential velocity of the growth steps increased. At concentrations of 0.085 M salt and 60 mg/ml protein, the maximum tangential velocity of the growth steps was 17.5 nm/s. No nucleation of any microcrystals from solution was observed. It is evident from Fig. 3, that solutions with low salt concentration have a greater capacity to produce large crystals than do solutions with high salt concentration. A similar influence of salt concentration on the growth kinetics of satellite tobacco mosaic virus crystals has also been observed [14].

Crystals grown from solutions of high salt concentration often exhibit pits on their faces. This may be attributed to the high sensitivity of the rate of two-dimensional nucleation in protein concentration. A small decrease in protein concentration at the center of a crystal face compared with the concentration at the crystal edges will lead to a larger difference in the rate of two-dimensional nucleation at the crystal center and crystal edges. At low salt concentrations, crystals grow without pits due to a decrease in the rate of two-dimensional nucleation, and reduced sensitivity to fluctuations in protein concentration.

Some experiments were also carried out where temperature was varied. Crystal growth ceased in solutions with concentrations of 0.09 M salt and 50 mg/ml protein at 38°C. In solutions with concentrations of 0.8 M salt and 5 mg/ml protein, crystal growth ceased at 50°C. Decrease in temperature to 2°C caused an increase in the rates of the nucleation and tangential growth step velocity.

These results imply that at constant temperature and precipitant concentration the crystal growth rate is dependent on variations in protein concentration in the anticipated manner, without altering molecular interactions, i.e. the growth rate is proportional to the protein concentration. Variation of precipitant concentration, however, changes interactions between protein molecules by changing the height of the potential barrier which a protein molecule must overcome for incorporation into the crystal [15]. In our experiments, an increase of precipitant concentration decreased the crystal surface free energy and, as a consequence, changed the growth mechanism from dislocation to two-dimensional nucleation.

The influence of the precipitant on the interactions between protein molecules can arise from two possible effects. First, salt ions may be able to attach themselves to protein molecules and participate in the formation of contacts between them. For example, for lysozyme solutions containing sulfate or phosphate it was found that these anions were adsorbed into the protein [16]. Bromide and chloride ions were also found to occupy well ordered positions at protein surfaces in lysozyme crystals grown from solutions containing these ions [17,18]. In our laboratory, sulfate ions were found to occupy well ordered positions on the surface of virions in satellite tobacco mosaic virus crystals. For crystals grown from NaI rather than $(NH_4)_2SO_4$, iodide ions occupied the same positions as did the sulfate ions (unpublished data). The other role of salt ions is screening protein surface electrostatic charges. Thaumatin crystals cannot be crystallized from solutions containing sodium chloride alone, nor from solutions of other salts, but thaumatin crystals will grow if only a small amount of Na or K tartrate is added to

virtually any salt solution. It was found by X-ray analysis [10], that tartrate ions participate in the crystal lattice, i.e. they must be present in solution to establish crucial connections between neighboring thaumatin molecules. Other salt ions simply create a screening effect.

Investigations of liquid–liquid phase separation [19,20] showed that the rate of crystal nucleation increases in the vicinity of a phase transition boundary. There is also a theoretical study [21], which indicates that crystals may nucleate and grow rapidly from protein rich droplets where the nutrient concentration is highly elevated. However, as was shown above, cessation of two-dimensional nucleation in solutions with high protein and low precipitant concentrations suggests that the protein rich phase is unlikely to be the source of crystal nucleation, but it can promote crystal growth if screw dislocations are present in the crystal.

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