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Macromolecular crystal growth investigations using atomic force microscopy

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Direct visualization of macromolecular crystal growth using atomic force microscopy (AFM) has provided a powerful tool in the delineation of mechanisms and the kinetics of the growth process. It has further allowed us to evaluate the wide variety of impurities that are incorporated into crystals of proteins, nucleic acids, and viruses. We can, using AFM, image the defects and imperfections that afflict these crystals, the impurity layers that poison their surfaces, and the consequences of various factors on morphological development. All of these can be recorded under normal growth conditions, in native mother liquors, over time intervals ranging from minutes to days, and at the molecular level.

Keywords: mechanism; screw dislocation; nucleation; protein crystals; defects.

AFM can yield images of uncommon clarity of intricate surfaces and specimens. It is applicable to areas ranging in size from less than 20 nm up to about 200 μm, and has a spatial resolution on soft materials of about 5 nm, with a height resolution as great as 0.5 nm. Thus it provides precise visual detail over a size range that is beyond most other technologies. Its application extends over the dimension range lying between single macromolecules, which can be studied by X-ray crystallography, macromolecular assemblies amenable to electron microscopy, and encompasses living cells which can just be visualized using light microscopy. Because scanning is carried out in a fluid environment, specimens suffer no dehydration, as is generally the case with electron microscopy. Specimens require no freezing, fixing or staining, indeed, living specimens can be observed over long periods so long as they stay relatively immobile. Subjects seem, in most cases, oblivious even to the presence of the probe tip passing over their surfaces.

The value of AFM, however, lies not just in its imaging capability, but in the non-perturbing nature of the probe interaction with the specimen surface. The specimen is unaware of the probe, and natural processes, such as growth, proceed. This allows an investigator to record not simply a single image, but a series that may extend over days. This is ideal for the study of the growth of macromolecular crystals, which develop over relatively long periods of time. Imaging frequency depends on the scan rate of the probe, and images may be gathered rapidly, generally within a few seconds or minutes. For protein crystal growth, a rather slow process, events on the surface impose no constraints on scan speed. Thus an extended series of high quality images are frequently accessible.

Another feature of AFM, when carried out in fluid cells, is that the media or mother liquor can be exchanged during the course of experiments without perceptibly disturbing the specimen. This is of considerable value in the study of protein crystals because it is often necessary to study growth processes under varying conditions of supersaturation. Growth steps are usually visible on the surfaces of crystals, and because advancement is relatively slow, their progression can be readily monitored in a temporal sequence of images. When rates are measured as a function of temperature, salt concentration, supersaturation, or some other parameter, then growth step velocities can be used to extract thermodynamic and kinetic parameters such as the step free energy, and the kinetic constant (Chernov et al, 1988; Chernov 1993; 1984). In optimal cases, individual virus particles, and even individual protein molecules can be observed as they are recruited into advancing step edges.

Macromolecular crystals grow by a number of mechanisms, some well known to conventional crystal growth (Buckley 1951; Burton et al. 1951; Chernov 1984), but also by another, probably unique mechanism. Although kinetic parameters are vastly different, the fundamental physics of the growth processes and their thermodynamic principles are the same as for other crystals (see Boistelle and Astier 1988; Feigelson 1988; Feher 1986; Durbin and Feher 1996). The major differences between protein and conventional crystal growth arise almost entirely from the large sizes and tenuous interactions of the macromolecules, the liquid environment and the important role of water, and the higher level of impurities that infect macromolecular samples.

What initially strikes the investigator employing AFM is the complexity, diversity, and variability of macromolecular crystal surfaces. These arise from the different mechanisms and growth phenomena, and their combinations, the array of defects and dislocations, the coarseness of the surfaces produced by impurities and multiple conformers, and the asymmetries and shapes arising from the bonding energies of molecules along different directions in the lattice. AFM has been utilized to observe not only the growth but also the dissolution of protein, nucleic acid, and virus crystals and this further serves to identify the sources of this diversity. AFM has also been used to provide quantitative descriptions of the crystallization process and to record the variety, density, and distribution of defects and dislocations throughout crystal lattices.

The purpose of an AFM investigation is first of all to increase our understanding of the fundamental physics and chemistry underlying the crystallization process. A second objective is to improve the crystallization process in support of protein X-ray crystallography. It is reasonable to expect that increased understanding of the process may ultimately be translated into more effective crystallization approaches and methods, and these into larger crystals of more proteins, as well as crystals that diffract to higher resolution, exhibit reduced mosaic spread, and better tolerate the rigors of cryocrystallography (Garman and Schneider 1997) and data collection.

There is a good likelihood that AFM may contribute in other ways to X-ray diffraction analyses. Because height information is preserved, for example, the handedness of molecular arrangements arising from screw axes can be deduced. Thus AFM may provide a means of discriminating enantiomorphs, as was done for crystals of fungal lipase (Kuznetsov, et al. 1997). Packing arrangements of macromolecules within unit cells are sometimes discernible, and this may assist in molecular replacement structure analyses. For virus crystallography, the contribution may be even greater. With AFM, orientations of individual virus particles, and even the capsomere structures of their surfaces may become visible (Malkin, et al. 1999). These can then be used to create starting models at low resolution for phase extension. It is important to note that images can be obtained under fully hydrated and unperturbed conditions, thus the images reflect the protein or virus particles, as they actually exist in the crystal.

A parameter that dominates virtually all aspects of crystal growth, protein and otherwise, is the degree of supersaturation of the mother liquor. Virtually all kinetic and thermodynamic parameters depend on supersaturation. This includes the probability of forming critical nuclei, i.e., the birth of a new crystal, initiation of new layers on existing surfaces, the velocity of step movements on surfaces,

incorporation of impurities and a host of lesser properties (Rosenberger et al. 1996; Schlichtkull 1957; Chernov 1984; Chernov, et al. 1988). Even the specific mechanism responsible for the growth of a crystal surface is dependent on supersaturation. Supersaturation, on the other hand, is itself a function of an array of experimental variables such as salt concentration, protein concentration, temperature, or other physical and chemical factors. It is also dependent on the physical and chemical features of the macromolecules and the manner by which they interact.

There are four principal mechanisms that, based on AFM, have been described for the development of faces for macromolecular crystals (Malkin et al. 1995). However, different faces of a crystal are generally non-identical and therefore, a crystal might simultaneously employ different mechanisms for development. Even a single face may depend upon more than one mechanism at the same time, and the type of mechanism may transform as some experimental variable, such as temperature is altered. Thus, when only limited observations of growth mechanism are available for a particular crystal, this does not necessarily mean that other mechanisms are not also operative. Most crystals, it appears, utilize all mechanisms at one time or another, though some one mechanism may be strongly preferred.

There are two dominant mechanisms in protein, nucleic acid, and virus crystal growth that produce growth step edges and thereby lead to layer addition of molecules. These are mechanisms that are also important in conventional crystal development. They are growth by screw dislocation, and growth by the spontaneous appearance on active surfaces of two-dimensional nuclei. Numerous examples have been recorded that illustrate both of these processes. Examples of screw dislocations on the surfaces of protein and virus crystals are shown in Figure 1, and examples of protein crystals growing by two-dimensional nucleation are shown in Figure 2. A third mechanism, known as normal growth does not lead to layer addition, but relies on intense random nucleation on active crystals where the surface free energy is unusually low. Though rare, this mechanism has been observed for several macromolecular crystals.

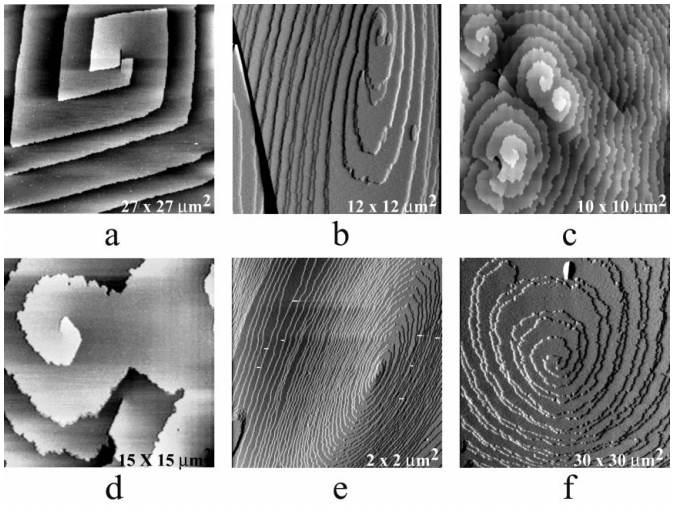

Figure 1 Slow dislocations on the surfaces of a variety of protein crystals as visualized in situ by atomic force microscopy, (a) canavalin, (b) lysozyme, (c) canavalin, (d) canavalin, (e) lysozyme, (f) trypsin.

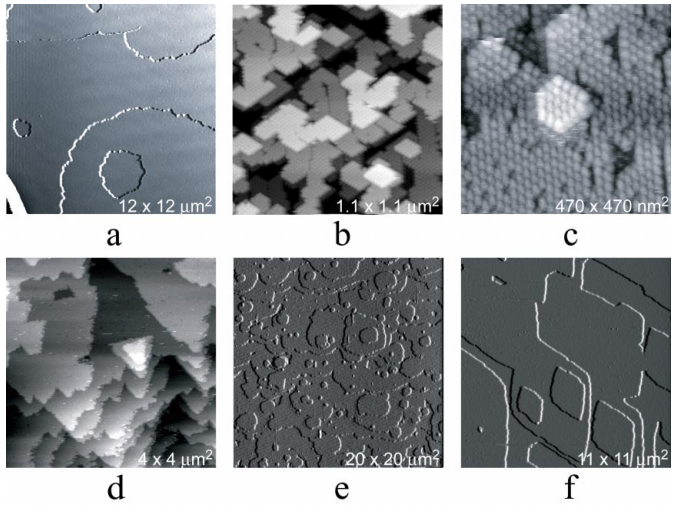

Figure 2 Two-dimensional nuclei on the surfaces of protein and virus crystals visualized by atomic force microscopy, (a) satellite tobacco mosaic virus (STMV), (b) cucumber mosaic virus (CMV), (c) STMV, (d) turnip yellow mosaic virus (TYMV), (e) thaumatin, (f) glucose isomerase.

A fourth mechanism, probably unique to macromolecular crystals, and one which has not been described for conventional crystal growth, arises as a consequence of the unique properties of concentrated macromolecular solutions. For virtually all of the protein, nucleic acid, and virus crystals investigated by AFM, the appearance of prominent, multilayer stacks of growth layers has been observed. Often these hillocks, whose characteristic shapes frequently reflect the gross morphology of the entire crystal, are ten to a hundred or more layers in height. Each layer of the stack provides step edges and, therefore, sources for tangential growth and the development of new layers. Crystal growth by this process, which has been termed growth by three-dimensional nucleation, can in some cases be the dominant mechanism (Malkin et al 1995).

A perplexing question is the origin of the multilayer stacks. An explanation, for which there is now good evidence, posits that they emerge from liquid protein droplets that populate concentrated macromolecular solutions (Asherie et al 1996; Lui et al 1995; Kuznetsov, et al. 1999), particularly within crystallization mother liquors. Liquid protein droplets are composed of hundreds to thousands of molecules, which exhibit short–range order mediated principally by non-specific hydrophobic interactions, and random arrangements of hydrogen bonds. Because of the extraordinary concentration of molecules in the droplets, they are locally hyper-saturated. When droplets contact crystal surfaces, the existing lattice serves as an epitaxial substrate to promote crystallization in the molecules above. These form a crystal layer, inspire crystallinity in the adjacent layer of molecules, and so forth, propagating a continuous series of growth layers, a multilayer stack.

The presence of such a liquid protein phase in concentrated protein solutions may also have implications for the physical chemistry and structure of concentrated macromolecular solutions, such as occur inside living cells. It may also provide a pathway, not only for the mechanism of crystal growth through three-dimensional nucleation, but also for the spontaneous formation in solution of critical nuclei (Ten Wolde and Frenkel 1997).

The levels of impurities in protein solutions, in spite of the great care normally shown by biochemists, vastly exceed those in conventional crystal growth solutions (McPherson et al 1996). This is unavoidable and unlikely ever to improve significantly. Intuitively, we might suspect that the contaminants most detrimental to macromolecular crystal growth would be those of large size, in the range of nutrient molecules or larger. These, if incorporated into a developing lattice would be most likely to produce dislocations, and the kinds of defects that can be seen using AFM. Probably the most deleterious impurities for a crystal are misoriented, improperly folded, or molecules having alternative conformations, including clusters or aggregates of the nutrient molecules, as well as foreign particles such as dust, microcrystals, and other contaminating macromolecules. AFM reveals that all of these kinds of impurities can readily be incorporated into protein and virus crystals.

Individual defects, and the overall defect structures (Tiller 1991) present in macromolecular crystals are particularly amenable to visualization by AFM (Malkin et al 1996). These show exceptional variety, and some are illustrated in Figure 3. Viewed as an ensemble of faults, they suggest the basis of the effect known to X-ray crystallographers as mosaicity. They also provide clues as to why some macromolecular crystals may be better ordered, and diffract to higher resolution than do others. Another important finding from AFM studies, because one can simply count defects and dislocations directly, is that macromolecular crystals contain two to four orders of magnitude more faults than do most conventional crystals (Malkin et al 1996; McPherson et al 1996).

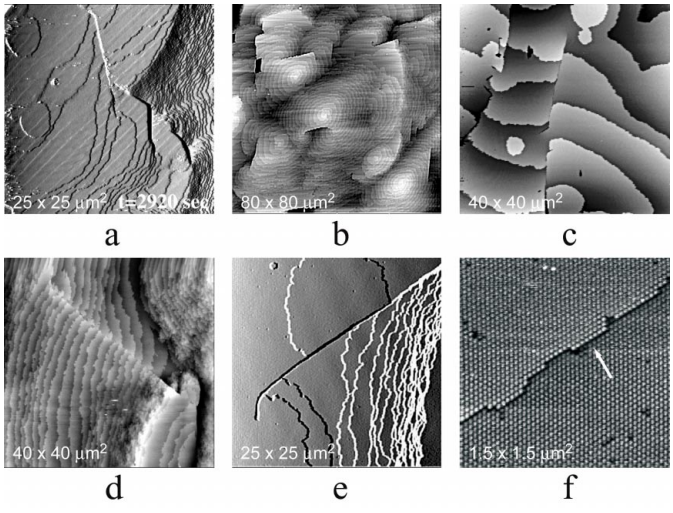

Figure 3 Stacking faults and other severe defects on the surfaces of some protein and virus crystals as visualized by atomic force microscopy, (a) STMV, (b) canavalin, (c) STMV, (d) trypsin, (e) STMV, (f) CMV.

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