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Mechanisms, kinetics, impurities and defects: consequences in macromolecular crystallization

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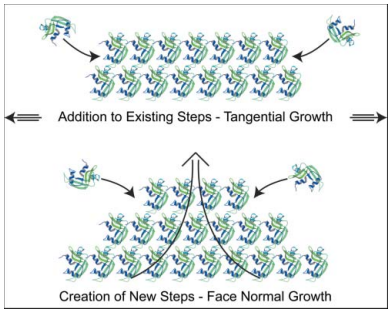
The nucleation and growth of protein, nucleic acid and virus crystals from solution are functions of underlying kinetic and thermodynamic parameters that govern the process, and these are all supersaturation-dependent. While the mechanisms of macromolecular crystal growth are essentially the same as for conventional crystals, the underlying parameters are vastly different, in some cases orders of magnitude lower, and this produces very different crystallization processes. Numerous physical features of macromolecular crystals are of serious interest to X-ray diffractionists; the resolution limit and mosaicity, for example, reflect the degree of molecular and lattice order. The defect structure of crystals has an impact on their response to flash-cooling, and terminal crystal size is dependent on impurity absorption and incorporation. The variety and extent of these issues are further unique to crystals of biological macromolecules. All of these features are amenable to study using atomic force microscopy, which provides direct images at the nanoscale level. Some of those images are presented here.

1. Introduction

There have been impressive advances in our appreciation of the physical properties of macromolecular crystals and how those properties have an impact on the X-ray diffraction data that we record from them. There has been parallel progress in understanding how protein, nucleic acid and virus crystals nucleate and grow. This new knowledge was founded upon more than a hundred years of research on the crystallization of ionic and conventional low-molecular-weight compounds, and the techniques and principles that emerged from that discipline. The complexity of macromolecules, which is extraordinary in itself, was superimposed upon what came before, and this amalgamation has generated a host of new ideas and directions. The role of water in crystallization, the large quantitative differences in diffusion rates, interfacial kinetics, thermodynamic parameters, the behavior of macromolecules in concentrated solutions, and the effects of ligands, ions and polymeric precipitants, have imposed themselves on investigators and become areas of significant interest.

Techniques that were developed for the study of conventional crystals, such as calorimetry, X-ray tomography and interferometry, have seen continued application in investigations of macromolecular crystallization, but they have been joined by new approaches such as static and dynamic light scattering, various kinds of optical microscopy, atomic force microscopy (AFM) and a range of biochemical technologies. Our recent advances in understanding macromolecular crystal growth we largely owe to the introduction and development of these novel technologies. We can attribute them as well to the vastly expanded investigator community, and the persistent, ever-increasing demand for more and better crystals for X-ray diffraction analysis.

Traditionally, macromolecular crystallization was viewed as having three phases: *nucleation*, generally the rate-limiting step; *growth*, the key to crystal perfection; and *termination of growth*, important to the ultimate crystal size. Understanding in all of these phases has progressed markedly, although we are still far from a comprehensive model and complete description of each. An attempt will be made in this article to summarize and briefly review what has been learned



about some crucial aspects of macromolecular crystal growth and to point out hopefully what remains to be done.

The reader should be advised, however, that there is much more to be said regarding the physics and chemical processes involved in macromolecular crystal growth than can be presented here. A good deal of this has been treated in more thorough and detailed reviews appearing previously. In particular, the excellent review by Vekilov & Chernov (2002) is very comprehensive and addresses the physics in quantitative, analytical terms, as do other papers by Chernov (Chernov, 2003; Chernov & Komatsu, 1995). The mechanisms responsible for macromolecular crystal growth and their visualization have also been reviewed by McPherson and coworkers (McPherson *et al.*, 1995, 2000, 2003; McPherson, 1989, 1999). The theory and physics of nucleation has been treated by Garcia-Ruiz (2003) and Vekilov & Chernov (2002), and the special problems associated with membrane-protein crystal growth by Caffrey (2003) and DeLucas (2009). Finally, an entire issue of the *Journal of Structural Biology* was devoted to macromolecular crystal growth (McPherson, 2003), as was an issue of *Methods* (McPherson, 2004).

2. The uniqueness of macromolecular crystals

Macromolecules, being unique in their properties, both in terms of size and complexity, give rise to crystals that are unique as well (McPherson, 1976, 1982, 1989, 1999). We cannot therefore expect that macromolecular crystals will necessarily develop according to precisely the same mechanisms or exhibit the same kinetic features as conventional crystals (Malkin, Kuznetsov & McPherson, 1996*b*; Malkin, Kuznetsov *et al.*, 1995; Feigelson, 1988; Rosenberger, 1986). Macromolecular crystals are relatively small in comparison with conventional crystals, rarely exceeding a millimetre on an edge in size and generally smaller. Because only one stereoisomer of a biological macromolecule naturally exists, they do not form crystals possessing inversion symmetry and therefore generally exhibit simple shapes that lack the polyhedral character of many conventional crystals. They are extremely fragile, often crushing upon touching; degrade outside a narrow temperature, ionic strength or pH range; generally exhibit weak optical properties and diffract X-rays to resolutions far short of the theoretical limit (McPherson, 1999). The reason for most of these sensitivities is that macromolecular crystals incorporate large amounts of solvent into their lattices, ranging from about 30% at the lower limit (*e.g.* insulin) to 90% or more (*e.g.* tropomyosin) in the most extreme cases (Gilliland *et al.*, 1994; McPherson, 1999; Gilliland, 1988). Proteins also have, as individual molecules, an array of water molecules that surrounds them. These are relatively fixed both in solution and in the crystal, and they mediate most intermolecular interactions in crystal lattices (Frey, 1994).

There are two other crucial differences between macromolecular and conventional crystal growth that produce important practical consequences. Firstly, macromolecular crystals are usually nucleated at extremely high levels of supersaturation, often several hundred to a thousand percent. Small-molecule crystals, on the other hand, usually nucleate at only a few percent supersaturation. Virtually every quantitative energetic and kinetic aspect of crystal growth is a direct function of supersaturation (Chernov, 1984; Vekilov & Chernov, 2002; Rosenberger, 1979). While high supersaturation may be essential to promote nucleation, it is far from ideal for growth, and many problems observed for macromolecular crystals reflect this. Furthermore, supersaturated macromolecular solutions, in addition to crystal nuclei, also produce alternate phases or solid states that we refer to collectively as amorphous precipitates, but that also include oils, gels and gums. Unlike most conventional systems, competition exists at both the nucleation and growth stages between crystals and precipitate. The rivalry is particularly acute because competition by precipitates and other phases is promoted by high levels of supersaturation. Because noncrystalline phases are kinetically favored, although higher energy states, they tend to dominate the nucleation process and often inhibit or preclude crystal formation.

Given the structural and dynamic complexities that afflict macromolecules, can we reasonably expect their crystallization to closely resemble that of conventional molecules? Most evidence suggests that the answer is, in principle, yes, but in practical terms, no. It appears that the fundamental mechanisms and pathways of macromolecular crystal growth are no different than for conventional crystals (Buckley, 1951; Burton *et al.*, 1951; Feigelson, 1988; Rosenberger, 1986) but that the magnitudes of the underlying kinetic and thermodynamic parameters that govern the process differ substantially.

Macromolecular crystals grow from solutions, as do most conventional organic molecule crystals, by what is classically referred to as sequential layer addition. Layer addition in the face normal direction (Fig. 1) relies on the generation of terraces and growth steps by two-dimensional nucleation and/or by spiral dislocations (Buckley, 1951; Burton *et al.*, 1951; Chernov, 1984; Rosenberger, 1979). It pictures the ordered addition of individual molecules at the resulting step edges, by tangential growth, at a rate determined by the level of supersaturation σ (Fig. 1).

Disorder in crystals, which limits diffraction resolution, is in part ascribed to the statistical spread of misorientation and lattice position of molecules from the mean, and also to the accumulation of impurities in crystals. The picture of disorder is made more realistic by inclusion of the concept of mosaicity (Cowley, 1984), which describes the real crystal in terms of ordered crystalline blocks or domains arising from defects. Ideally, the growth surfaces and step edges should be relatively smooth, with defects, dislocations and step-edge roughening principally consequences of the incorporation of impurities.

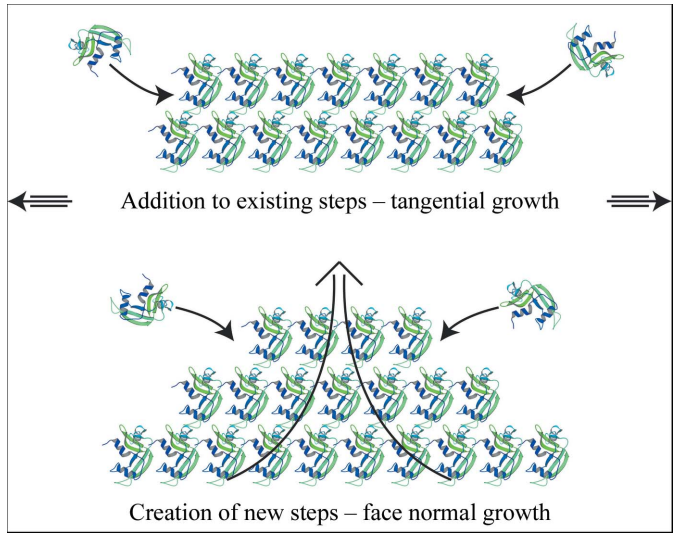


Figure 1
Tangential and face normal growth are here shown schematically. Tangential growth occurs by the addition of molecules to step edges, causing the lateral extension of surface layers. Face normal growth proceeds by the addition of nascent layers atop those already present. Face normal growth requires two-dimensional nucleation on the crystal surface and therefore must overcome some energy barrier. Thus, face normal growth is generally the rate-limiting step of crystal growth, and it may cease well before tangential growth.

3. Nucleation of macromolecular crystals

At high levels of supersaturation, far from equilibrium (*i.e.* saturation), molecules continuously associate to form clusters and aggregates having varying degrees of order (Rosenberger, 1979; Haas & Drenth, 1999; Feher, 1986; Feigelson, 1988; Boistelle & Astier, 1988; Feher & Kam, 1985; Rosenberger *et al.*, 1996). The size of an individual cluster can tacitly be defined by a radius R. Molecules free in solution are continually recruited into potential nuclei, while others dissociate. If at some point R exceeds some critical value R_c (Oxtoby & Kashchiev, 1994; Kashchiev, 2000), then it becomes energetically favorable for the aggregate to accumulate new molecules more rapidly than to lose old molecules, and a crystal nucleus will be born. The higher σ, the greater the probability that molecules will be gained rather than lost to the aggregate, and the smaller is R_c. The energy (or probability) barrier to achievement of critical nuclear size R_c (Fig. 2), therefore, is supersaturation-dependent (Chernov, 1984; Rosenberger, 1979; Vekilov & Chernov, 2002).

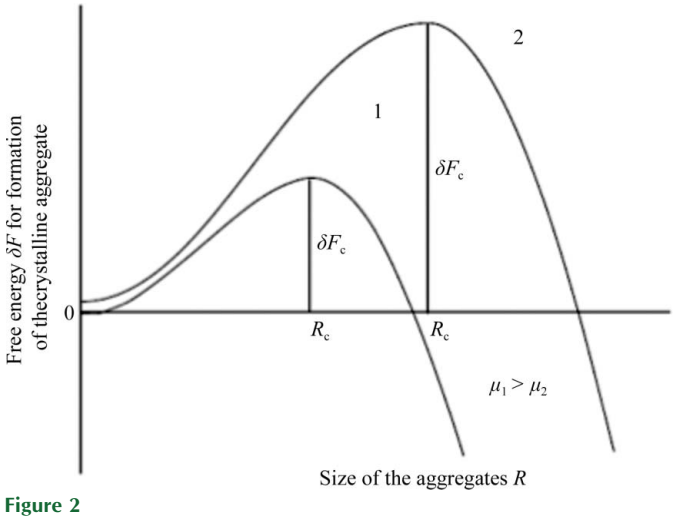


Figure 2
Dependencies of δF, the free energy, on the radius R_c of a critical nucleus for two values of solution supersaturation σ_1 and σ_2. Peak 1 corresponds to higher supersaturation and therefore requires a smaller, ordered cluster to create a critical nucleus. Peak 2, at lower supersaturation, demands a larger aggregate size for successful formation of a critical nucleus.

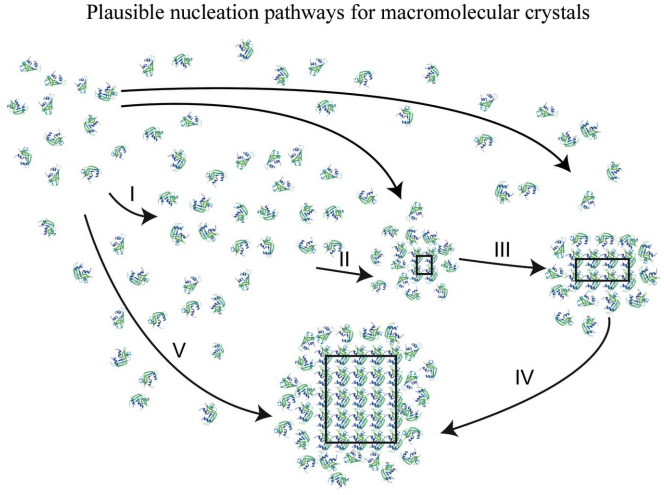


Figure 3
Illustrated here is the concept of crystal nucleation and development from an initial 'molten globule' aggregate. As the supersaturation is increased and association of molecules is promoted, molecules self-organize into larger disordered aggregates. The interactions are principally nonspecific, transitory, van der Waals interactions that permit dynamic flexibility. The process is also driven by the release of water molecules from the hydrated macromolecules. Over time the cores of aggregates, essentially sequestered from solvent, reorient, redistribute and give way to more geometrically rigorous hydrogen bonds and electrostatic interactions (II). Formation of the latter tends to order and stabilize the aggregate cores, which subsequently increase in size to produce a critical nucleus (III). Molecules free in solution are then absorbed to the crystal surface and increase its size by incorporation into the lattice (IV) to produce the mature crystal.

The exact nature of the nucleus and the detailed process by which R_c is attained (Garcia-Ruiz, 2003; Vekilov & Chernov, 2002; Yau & Vekilov, 2000) remains controversial (Fig. 3). Whether the critical nucleus is initially and continuously ordered, or whether it attains order through restructuring of an aggregate initially lacking long-range order, is not certain, nor is whether it forms by the coalescence of arbitrary subnuclear clusters or by strict monomer or oligomer addition, or by all of these simultaneously or sequentially.

We have some reasonable hypotheses regarding the differences between assemblies of molecules that serve as critical nuclei and aggregates that evolve into amorphous material. Within crystal nuclei the macromolecules tend to make interactions with one another in all three directions in a periodic manner. Amorphous precipitates, on the other hand, tend toward linear arrangements with arbitrary branching (Feher & Kam, 1985). Studies based on quasi-elastic light scattering (QELS) suggest that crystal nucleation and the formation of amorphous precipitate are distinct and can be discriminated prior to the appearance of visible crystals (Wilson, 2003; Feher & Kam, 1985; Malkin *et al.*, 1993; Malkin & McPherson, 1993*b*, 2004; Kam *et al.*, 1978; Mikol *et al.*, 1990, 1991; George & Wilson, 1994). Some investigators postulate transition from an amorphous or fractal state into a crystal nucleus (Georgalis *et al.*, 1993). Other work demonstrates that conditions propitious for crystal growth may be predicted from properties of the mother liquor that are identifiable by QELS or static light scattering (Kadima *et al.*, 1990; Kam *et al.*, 1978; McPherson *et al.*, 1995; George & Wilson, 1994; Baldwin *et al.*, 1986; Mikol *et al.*, 1990, 1991). A more thorough review of light-scattering investigations, their findings regarding nucleation, and their value in predicting useful crystallization conditions is contained in the article by Wilson and DeLucas in this series.

A model that is consistent with most observations is that macromolecules initially associate through van der Waals or other nonspecific interactions to produce a quasi-stable, fluid aggregate having only short-range order, as do the amino acids within the molten

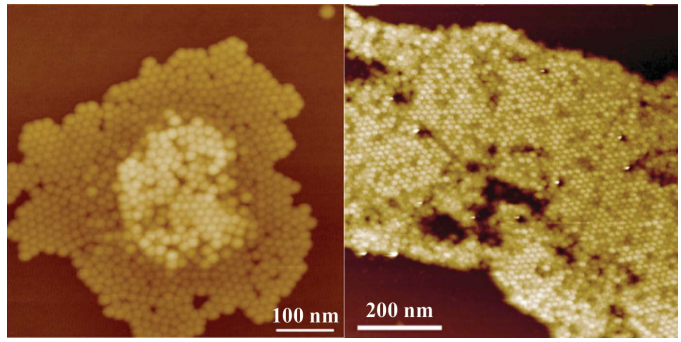


Figure 4
If icosahedral virus particles are simply dried slowly upon a substrate, then they organize themselves into sheets, sometimes multilayer, having, in these AFM images, apparent crystalline order. The arrays are not truly crystalline, however, as the individual particles exhibit arbitrary orientations inconsistent with crystallographic symmetry. It is not hard to imagine, however, that by gradual rotation of particles and minor restructuring that a crystal nucleus could emerge. On the left is a pool of *Brome mosaic virus* (BMV) and on the right *Satellite tobacco mosaic virus* (STMV).

globule theorized for nascent polypeptides as they fold into a fully ordered state (Ohgushi & Wada, 1983). The model may also be exemplified by the pools of virus particles condensed upon a substrate and visualized by AFM in Fig. 4. Though the virus particles are translationally periodic, they initially have arbitrary orientations and are not crystalline in the strict sense. Eventual reorientation and rearrangement then proceeds in the unique internal environment of the fluid aggregate, accompanied by the formation of more geometrically rigorous hydrogen and electrostatic bonds, to produce a three-dimensionally ordered core, again analogous to a folded protein. For such a phase transition, R_c may have a more complex meaning than we currently ascribe to it, specifying, possibly, the initial size of the disordered aggregate, the size of an eventually self-propagating, ordered core, or some combination thereof.

Estimates of R_c as a function of σ have been obtained (Fig. 5) for some proteins and icosahedral viruses using QELS (Malkin *et al.*, 1993; Malkin & McPherson, 1993*a*,*b*). QELS can provide σ-dependent, quantitative descriptions of aggregation pathways that ultimately lead to the formation of critical nuclei. In addition, QELS allows measurement of the rates of growth of nuclei until they reach a size visible with a light microscope, and makes possible the calculation of estimates of R_c as a function of σ (Fig. 5).

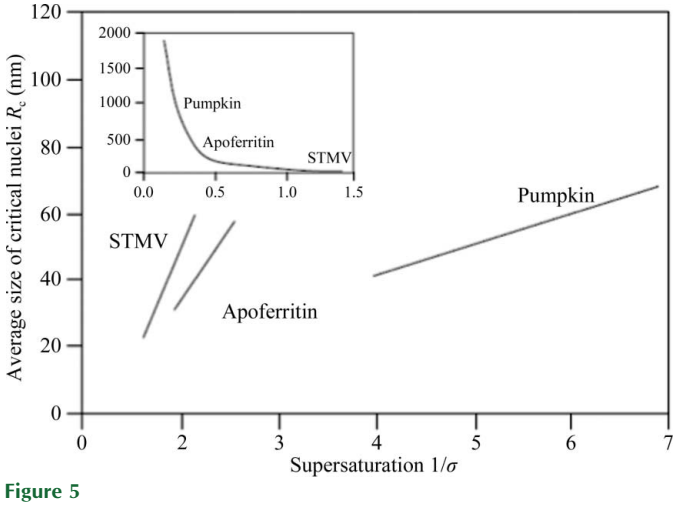


Figure 5
Dependencies of the critical nuclear size R_c *versus* inverse supersaturation, $1/\sigma$, for pumpkin globulin, apoferritin and STMV crystallization. The corresponding values of the interfacial energies α and molar surface energies are pumpkin globulin, $\alpha = 6.1 \times 10^{-9}$ J cm^{-2}; apoferritin, $\alpha = 2.7 \times 10^{-9}$ J cm^{-2}; STMV, $\alpha = 1.8^{-9}$ J cm^{-2}. The inset shows the supersaturation dependency of the number of protein molecules or virus particles comprising the critical nucleus.

A salient question is what dimensions a nucleus must have in order to persist and to develop into a crystal. Nucleation represents a phase transition, and is therefore of significant interest beyond crystallization. Critical nuclear size is dependent on the particular molecule, the intermolecular interactions driving crystallization and, as noted above, on the degree of supersaturation σ (Chernov & Komatsu, 1995; Garcia-Ruiz, 2003; Vekilov & Chernov, 2002).

While it is difficult to use AFM to study the formation of three-dimensional critical nuclei directly from solution (homogeneous nucleation), the two-dimensional nuclei that form on existing crystal surfaces and give rise to new growth layers (Fig. 1) have been visualized and quantitative estimates of the size of R_c have been extracted. Examples are shown for thaumatin crystals in Fig. 6. Because these share the properties of nuclei that initiate new crystals, their analysis is also of considerable interest. Using AFM and simply estimating the number of molecules or unit cells comprising potential two-dimensional nuclei, and evaluating whether they persist over time or disappear, and carrying out these observations at different supersaturations, the sizes of critical nuclei as a function of supersaturation can be estimated. This has been performed for several crystals, and has yielded some important quantitative information that can then be related to the bonding energies and assembly properties that govern nucleus formation (Chernov & Komatsu, 1995; Malkin, Kuznetsov *et al.*, 1995; Vekilov & Chernov, 2002).

4. Application of AFM to macromolecular crystal growth

AFM has proven to be a particularly powerful and insightful technology for investigating the crystallization of proteins, nucleic acids and viruses (McPherson *et al.*, 2000, 2001). Unlike most other high-magnification visualization methods, AFM allows observation of growing crystals, *in situ*, in their mother liquors. It is non-destructive and so far as we can discern, virtually unobtrusive. Thus, the growth of a crystal can be recorded continuously or periodically over hours, or even days, at relatively brief intervals of 1–5 min. Because virus and other macromolecular crystals develop at such a slow pace compared with conventional crystals, at least an order of magnitude lower, the kinetics of processes can be measured precisely. The mechanisms of growth can be visualized in some cases, and events involving only a single molecule or virus particle can be recorded. With AFM, defect structure is revealed that cannot be detected even by X-ray diffraction or X-ray topography (Kuznetsov *et al.*, 1995; Malkin *et al.*, 2006).

The advantages of AFM for investigating macromolecular crystal growth are dramatically increased by its application to virus crystal

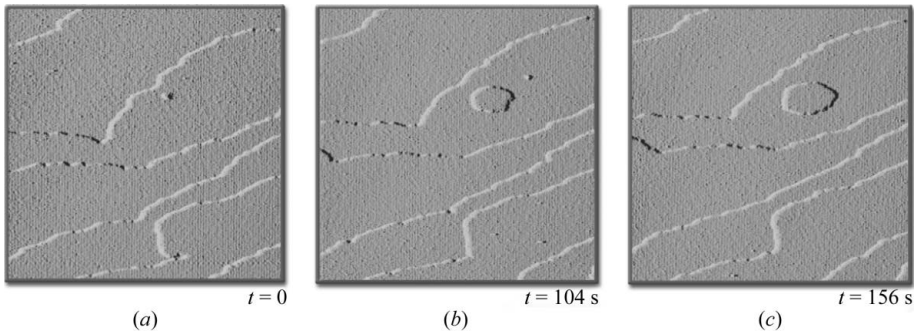


Figure 6
In (*a*) a very small molecular cluster appears on the surface of a growing thaumatin crystal. It persists for a time into (*b*), but has dissolved by the time of (*c*). In (*b*) a larger two-dimensional nucleus appears as well and continues to develop into the larger two-dimensional island seen in (*c*). The critical nuclear size R_c therefore lies somewhere between R for each of the two nuclei. Scan areas are 15 × 15 µm.

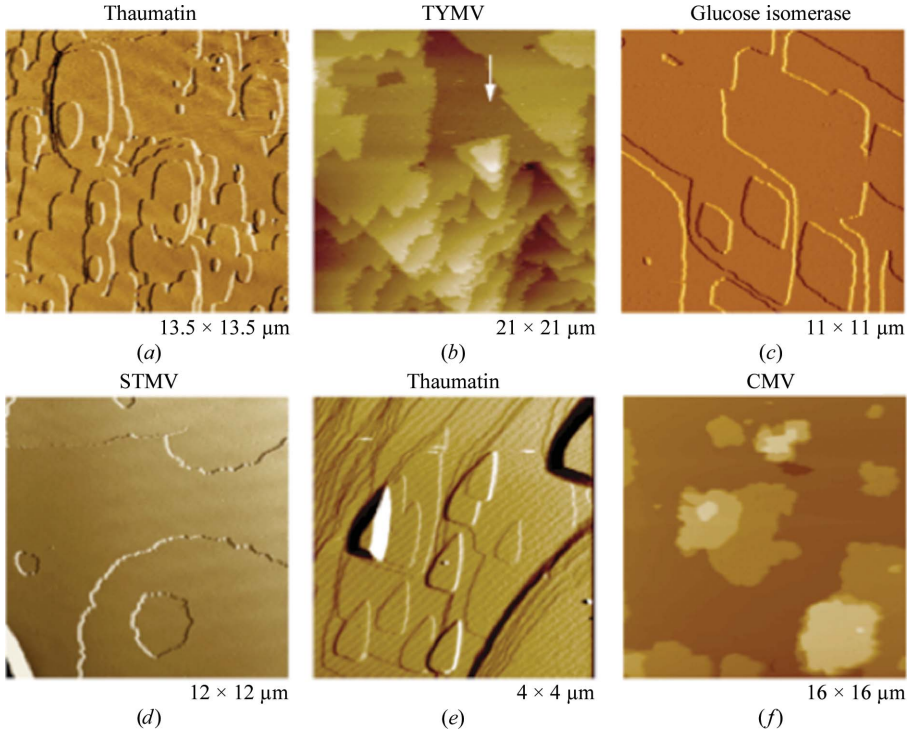


Figure 7
A major source of the growth steps and layers on the surfaces of growing macromolecular crystals, particularly at medium to high levels of supersaturation, are two-dimensional nuclei that exceed the critical nuclear size and subsequently develop into two-dimensional islands. Shown here are two-dimensional islands on a variety of protein and virus crystals. This is the dominant mechanism for face normal growth for most macromolecular crystals. In (*b*) the arrow denotes a triangular nucleus that reflects the symmetry of the crystal face.

growth. The growth of icosahedral virus crystals, as best we can determine, parallels that of more conventional protein crystals in virtually every way. Virus particles that form crystals, however, are of relatively large size, about 17 nm in diameter for $T = 1$ particles, where T is the triangulation number (Caspar & Klug, 1962), and about 30 nm diameter for $T = 3$ virions. Many of the AFM images in this article are of growing *Satellite tobacco mosaic virus* (STMV; $T = 1$, 17 nm diameter particles) crystals, and crystals of *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV) and *Turnip yellow mosaic virus* (TYMV), where $T = 3$, with 28–30 nm particle diameters. For these crystals, individual particles can easily be seen as they join a step edge or leave a crystal surface. Thus, single-particle (or virion) kinetic measurements are possible (Malkin *et al.*, 1997, 1999, 2001; Kuznetsov *et al.*, 1999, 2000).

Application of AFM currently provides an unmatchable approach for the study of aggregation, nucleus formation, crystal growth and dissolution, and the wide variety of physical and chemical phenomena that affect the process. This applies not only to the formation of critical nuclei, but nucleation on heterogeneous surfaces, as well as two-dimensional nucleation (Fig. 6) on the surfaces of growing crystals (Malkin, Kuznetsov *et al.*, 1995; Malkin, Land *et al.*, 1995). No attempt to describe the detailed principles and operation of AFM will be given here, as the technology and its application to macromolecular crystal growth has been thoroughly reviewed elsewhere (Kuznetsov *et al.*, 1997; McPherson, 1999; McPherson *et al.*, 2000, 2001).

5. Mechanisms of macromolecular crystal growth

As illustrated in Fig. 1, there are two processes that must occur for full acquisition of an additional surface layer. These are termed face normal growth and tangential growth. Growth normal to the surface proceeds by the creation of nascent layers, or islands in most cases, which exhibit step edges to which new molecules can be added tangentially. Because addition of new layers requires, in the absence of dislocations (see below), the spontaneous appearance of a new ordered arrangement of molecules where none previously existed, *i.e.* atop the previous surface layer, it is another nucleation process. Nucleation events, which require the creation of order, are generally unfavorable in a kinetic sense and require the system to surmount an energy (or probability) barrier. Thus, they are usually the rate-limiting step in most physical and chemical processes, including crystal growth. The initiation of new step edges and layers is then the slower, more demanding process in crystal growth.

Tangential growth refers to the recruitment of molecules into step edges and the extension of new layers over the surface. This is, relatively, a much easier process because the incorporation of a new individual is essentially a cooperative process favored by both molecules composing the existing step edge and the new recruit. The energetics of incorporation favors the union. Thus, once a nascent layer appears, its two-dimensional expansion may proceed relatively unimpeded, except by impurities (see below). Indeed, if we look at the surfaces of a crystal that have stopped growing one sees that there are no islands or step edges remaining on the surface; it is flat. While the last available step edges have expanded over the surface to the very frontiers of the crystal faces, the barrier to the formation of new layers, the two-dimensional nucleation barrier, could not be overcome.

A principle that dominates virtually all aspects of crystal growth, macromolecular and otherwise, that again deserves emphasis, is the degree of supersaturation σ of the mother liquor. Virtually all kinetic and thermodynamic variables are directly dependent upon supersaturation (Chernov, 1984; Rosenberger, 1979; Vekilov & Chernov, 2002). This includes the probability of forming critical nuclei, that is

the birth of a new crystal, initiation of new layers on an existing surface, the velocity of step movement on the surface (tangential growth), the incorporation of impurities (Rosenberger, 1979; Rosenberger *et al.*, 1996; Schlichtkrull, 1957; Chernov, 1984; Chernov *et al.*, 1988; Vekilov & Chernov, 2002), and a host of lesser properties. The particular mechanism employed for growth on a crystal surface is also critically dependent on supersaturation. Supersaturation in turn is a function of a collection of experimental variables such as salt concentration, macromolecule concentration, temperature, pH or other physical and chemical factors. It is also dependent on the underlying physical, chemical, conformational and dynamic properties of the macromolecules and the manner by which they interact with one another.

There are four principal mechanisms that have been described for the layer growth of faces of macromolecular crystals (Malkin, Kuznetsov *et al.*, 1995; McPherson, 1999; McPherson *et al.*, 2000, 2001). It should be noted, however, that different faces of a single crystal, being non-identical, might simultaneously employ different mechanisms for development. Even a single face may use more than one mechanism at the same time, and the type of mechanism may change as some experimental variable, such as temperature, is altered. Thus, when only one, or a few, observations of a growth mechanism is available for a particular crystal, this by no means implies that other mechanisms are not involved at other times or under other conditions. Most crystals, it seems, utilize all mechanisms at one time or another, although one particular mechanism may be strongly favored. Virus crystals may be an exception.

Over a broad range of supersaturation, most protein and virus crystals generate step edges and new growth layers through a process of two-dimensional nucleation on existing surfaces. Undoubtedly guided by the underlying ordered pattern (homoepitaxy), molecules from solution adhere to the surface and organize themselves into crystalline arrays consistent with the supporting lattice. Molecules are also free to leave the surface, but when the organized array reaches some critical size the balance favors addition, and it persists and expands as a growth island by recruitment of additional molecules

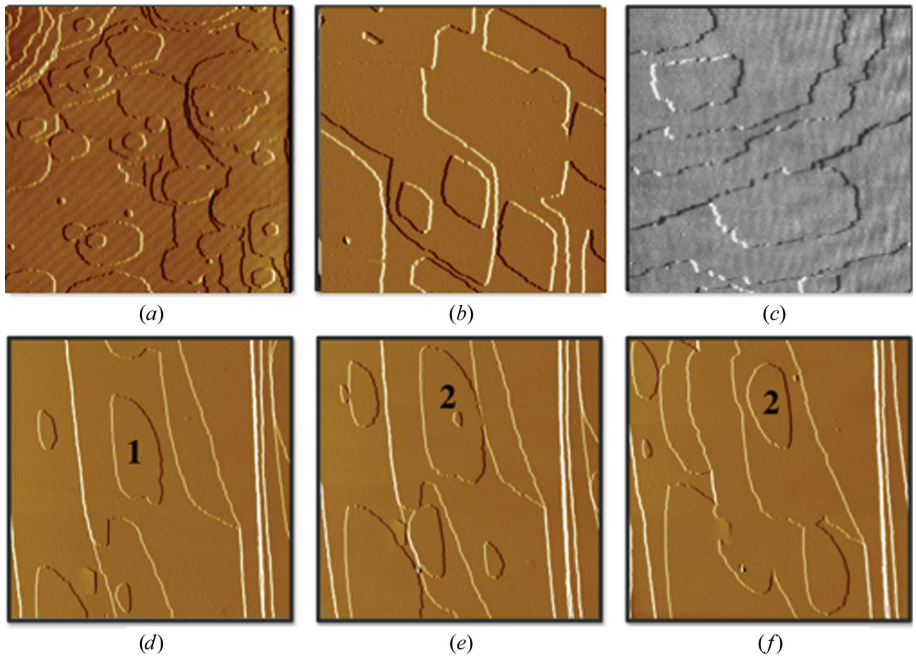


Figure 8
(*a*), (*b*) and (*c*) are examples of two-dimensional islands on crystal surfaces that grow tangentially and merge seamlessly to form new layers. The 111 faces of (*a*) tetragonal thaumatin, (*b*) orthorhombic glucose isomerase and (*c*) orthorhombic catalase are shown. (*d*), (*e*) and (*f*) show successive AFM images following the appearance of a two-dimensional island (labeled 2) on the surface of another island (labeled 1) of a growing beef liver catalase crystal. The islands are asymmetric with a curved side and a flatter side. Islands forming successive layers display alternating orientations related by 180°, reflecting the twofold screw axis perpendicular to the crystal surface. Scan areas are (*a*) 15 × 15 µm, (*b*) 11 × 11 µm, (*c*) 17 × 17 µm and (*d*–*f*) 32.5 × 32.5 µm.

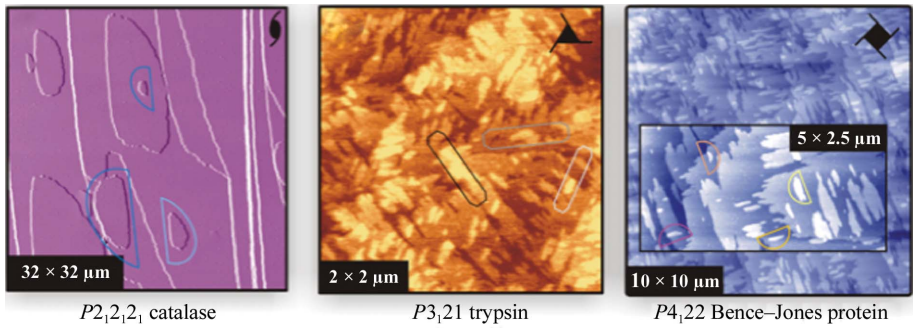


Figure 9
The symmetries of crystals are reflected in their overall morphology as well as in the growth patterns on their faces. In the cases of screw axes, this takes the form of successive growth layers that have a symmetry relationship determined by the order of the screw. For an *n*-fold screw axis, there are *n* related layers which are related by a 360°/*n* rotation.

into its step edges (Fig. 6). The first event, two-dimensional nucleation, provides growth normal to the surface, and the latter events provide tangential growth. Crystals growing by this process of two-dimensional nucleation generally exhibit growth islands abundantly scattered over their surfaces, as seen in Fig. 7. In many cases, the shapes of the growth islands reflect the geometries of the morphological faces on which they are present, as also illustrated by Figs. 8 and 9. AFM allows an investigator to observe changes in the islands as a function of time, thereby permitting direct calculation of step movement rates, which at specified supersaturations then allow the deduction of important thermodynamic and kinetic parameters (Vekilov & Chernov, 2002; Durbin & Feher, 1996; Kuznetsov *et al.*, 1999; Malkin, Kuznetsov, Glantz *et al.*, 1996; Plomp *et al.*, 2001).

Using AFM, the heights of steps on surfaces can usually be measured to a precision of a few angstroms, and frequently they correspond to a single unit-cell dimension. This implies that many crystals grow by initiating and completing discreet crystallographic unit cells rather than starting and filling cells here and there on the island. Tetragonal thaumatin crystals (Ko *et al.*, 1994), for example, are in this category (Malkin, Kuznetsov, Glantz *et al.*, 1996). The question as to whether molecules add individually to the advancing step edge, or by ordered aggregates corresponding to an entire or discrete portions of a unit cell, can be answered by examining changes in the fine structure of step edges in high-magnification, high-resolution images. This has been performed in the case of thaumatin crystals, where it was found that the step edges do advance by the incorporation of individual molecules and not by the addition of preformed assemblies or clusters (Kuznetsov *et al.*, 1998; Kuznetsov, Malkin *et al.*, 1999). This latter process could occur in some instances, but it appears not to be common.

Thermodynamic considerations would suggest that for mixed solid and fluid phases, molecules would be expected to leave the crystalline state and return to the solution phase while others do the opposite. If the system is undersaturated, the first process would dominate and the crystals would dissolve, while dominance of the latter process would lead to net growth. At equilibrium, no net change would occur. Kinetic considerations, however, could prevail, as they frequently do in chemical systems, depending on the energy barriers to adding molecules or removing them from the lattice. Because molecules at step edges make the fewest contacts with neighbors, most of the coming and going would be expected to occur there. Studies of surface development and step-edge movement of a number of protein crystals under a variety of conditions, however, suggest that once a molecule joins the lattice at a step edge, if the solution is at all supersaturated, it virtually never leaves (Kuznetsov *et al.*, 1998, 1999). There is, in fact, little or no coming and going. For some crystals this is extreme. Catalase crystals grown from PEG, and STMV crystals grown from ammonium sulfate, for example, remain virtually insoluble once grown, even when placed in pure water. AFM suggests that it is, in most cases, indeed difficult to pull a macromolecule free of its crystal lattice, and that the energy barrier to doing so is substantial.

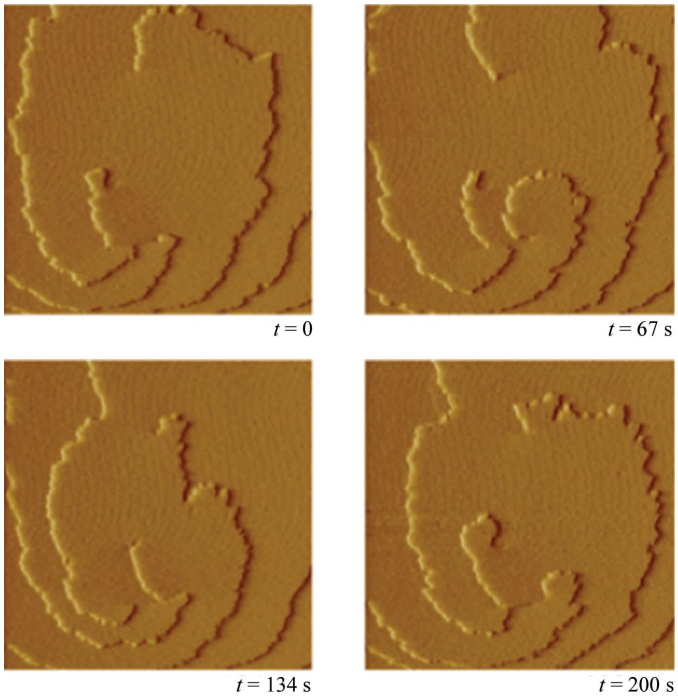


Figure 10
A series of AFM images shows the development over time of step edges around a complex screw dislocation source on the surface of a growing trypsin crystal. Scan areas are 10 × 10 μm.

Growth islands are not always a single crystallographic unit cell in height, however, and some interesting examples have been recorded (Plomp *et al.*, 2002; Malkin *et al.*, 2006). Orthorhombic crystals of beef liver catalase develop by the formation of growth islands corresponding in height to exactly one half of a unit cell (Malkin *et al.*, 1997). Examination of the X-ray crystallographically determined structure (Ko *et al.*, 1999) provides an explanation. Molecules in the **a** × **b** plane interact closely with one another to form tight arrangements related by 2_1 screw axes along **a** and **b**. Molecules related by the 2_1 axes along **c**, however, are separated from one another by solvent and interact only weakly. Thus, layers of molecules corresponding to the 'bottom halves' and 'top halves' of unit cells are deposited alternately along **c**. Because the 'bottoms' and 'tops' have different orientations, owing to the 2_1 screw axis relationship (note the curved and flat sides of the islands on catalase crystals in Fig. 8, for example). they can be readily identified in the images. Similarly, examples where islands corresponding to sequential layers are elongated perpendicular to threefold and fourfold screw axes have also been recorded (Plomp *et al.*, 2002; Fig. 9).

Two-dimensional islands generally do not advance at equal rates in all directions. This is the case because the step edges present a different appearance; that is, they display different bonding possibilities in different directions. Thus, new molecules are recruited differentially into step edges at different points on the island boundaries, and therefore at different rates. Impurities, which also affect the rate of step advancement, are also incorporated with different affinities around the growth islands, and these too affect recruitment rates, leading in turn to asymmetric shapes for the islands, such as those seen for catalase crystals, for example (Figs. 8 and 9).

Another mechanism common to crystals of conventional molecules is growth through the creation of step edges at screw dislocations in the lattice (Buckley, 1951; Burton *et al.*, 1951; Chernov, 1984; Rosenberger, 1979; Frank, 1949). These arise when, for one reason or another, perhaps the incorporation of a contaminant or the misincorporation of one or several molecules, a displacement occurs along the direction normal to the surface. At such points, steps are continuously propagated as multiple layers spiralling about the dislocation, hence the name screw dislocations (Fig. 10). The important difference between growth by this mechanism and growth through the formation of two-dimensional islands is that face normal growth is significantly facilitated since no nucleation step is necessary to initiate a new layer. The continuously generated step edges from a

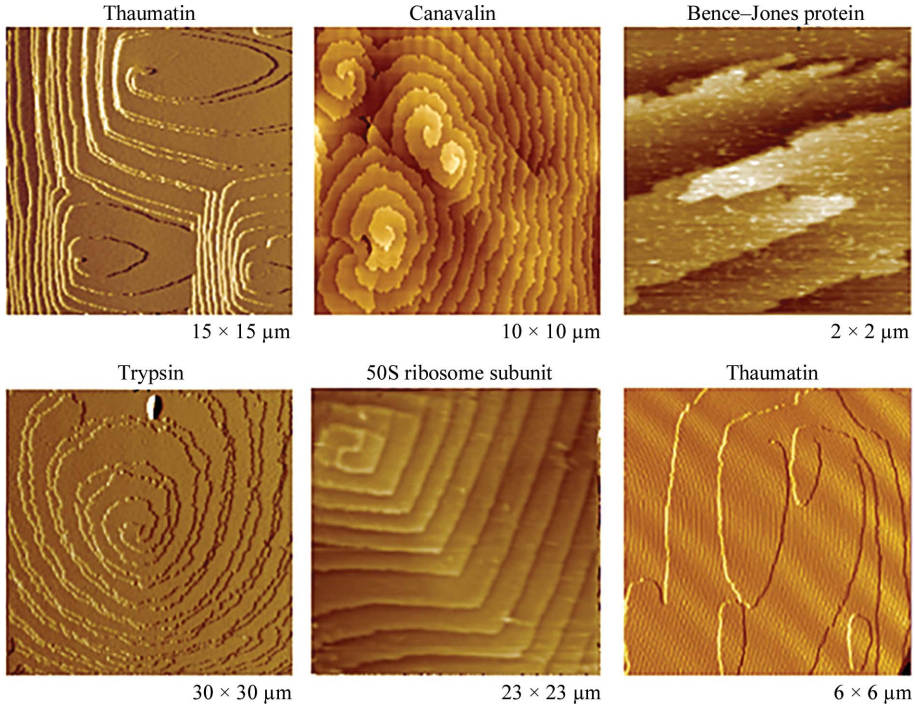


Figure 11
A major source of growth steps on growing crystals, particularly at lower supersaturation, are screw or spiral dislocations. Shown here are a variety of screw dislocations on the surfaces of macromolecular crystals that illustrates their diverse character.

screw dislocation provide nascent layers and the crystal grows almost exclusively by tangential addition of molecules to step edges.

An array of screw dislocations from various crystals is shown in Fig. 11. In most cases, the spirals, like the growth islands discussed above, are asymmetric in shape, again reflecting the symmetry properties of the specific crystal. Spirals can be left-handed or right-handed, and a single crystal surface will often exhibit both. Spirals may also be single or double at the dislocation, and these more complex spirals may again have either hand. Although screw dislocations have been observed on the surfaces of nearly all of the protein and nucleic acid crystals examined, and even on crystals of ribosomal subunits, they have not been identified on any virus crystal. Not all of the former kinds of crystals have an equal propensity to form screw dislocations, possibly owing to differences in mechanical or material properties. Some crystals, like those of beef liver catalase, have virtually none, while the surfaces of rhombohedral canavalin crystals are crowded with them. The appearance of screw dislocations, however, appears to be crystal-dependent rather than molecule-dependent. While merohedrally twinned rhombohedral canavalin crystals (Ko *et al.*, 2001) are thick with screw dislocations, the non-twinned orthorhombic and hexagonal forms are not.

On many crystals, particularly at high supersaturation where growth is rapid and step advancement tends to become disorganized, large, imposing macrosteps (Vekilov *et al.*, 1997), such as those captured in Fig. 12, consisting of stacks of growth layers are common. Although individual layers grow independently by molecular addition to their step edges, except through competition of their diffusion fields, the macrosteps tend to move like coherent waves over the surfaces of crystals. The remarkable thing is that when the growth layers of one macrostep encounter those of another, the corresponding individual layers comprising the two macrosteps merge and form an apparently flawless union.

Macromolecular crystals, as noted above, grow by the addition of uniform layers, one atop another, through addition of molecules to the edges of the layers. As seen above, these layers may be generated by two-dimensional nucleation or by screw dislocations. Another mechanism for crystal development, however, called normal growth, does not proceed by layer addition, but by random recruitment of molecules at arbitrary sites on the surface. In a sense, molecules join the lattice everywhere on the surface in a random manner. Essentially, the energy barrier for addition of new layers in the face normal direction is so low, or supersaturation is so high, that face normal growth is no longer kinetically limiting and becomes competitive with tangential growth. New islands form atop pre-existing ones before any island has an opportunity to expand. This kind of crystallization is characteristic, for example, of conventional crystals grown from the melt. It leads to an atomically 'rough' surface as opposed to the atomically 'smooth' surface yielded by layer growth.

Macromolecular crystals have been observed by AFM to grow by this normal mechanism, in which cases the surface appearance

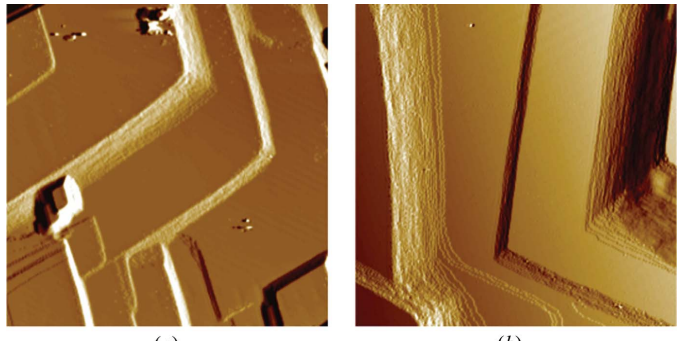


Figure 12
Macrosteps comprised of many tens of individual layers are seen on the surface of this lysozyme crystal. The macrosteps are produced by a phenomenon known as step bunching (Vekilov *et al.*, 1997; Vekilov & Rosenberger, 1998), often a consequence of thermal perturbation, or some other disruption, occurring during rapid growth. Scan areas are (*a*) 70 × 70 μm and (*b*) 45 × 45 μm.

becomes extremely rough and irregular, as shown for the apoferritin crystals in Fig. 13. Growth by this mechanism in the regime of high supersaturation is completely disorganized and produces, as one might anticipate, crystals of very poor quality, although their ultimate size is not necessarily restricted. Apoferritin crystals, for example, grow to very large sizes.

A mechanism that may be unique to macromolecular crystals is illustrated in Fig. 14. It has not been described for conventional crystal growth, and it may arise as a consequence of the unique properties of concentrated macromolecular solutions (Asherie *et al.*, 1996; Liu *et al.*, 1995; Rosenbaum *et al.*, 1996; Haas & Drenth, 1999; Kuznetsov *et al.*, 1998). For virtually all of the protein, nucleic acid and virus crystals investigated by AFM, the sudden appearance of vast, multilayer stacks of growth layers has been observed. Often, these hillocks (or mesas), whose characteristic shapes frequently reflect the gross morphology of the entire crystal (Fig. 15), 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 formation of new layers. Growth by this mechanism, which has been termed growth by three-dimensional nucleation, can in some cases be a dominant growth mechanism (Malkin, Kuznetsov *et al.*, 1995, McPherson, 1999). It is noteworthy that when tangential growth of layers proceeds simultaneously from multiple, proximal multilayer stacks on the surface of a crystal, the corresponding layers from the various stacks ultimately encounter one another, merge and knit with one another in a contiguous manner.

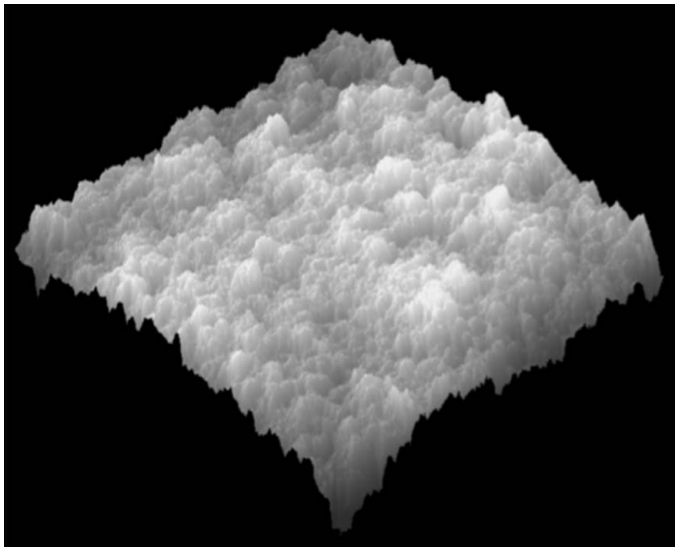
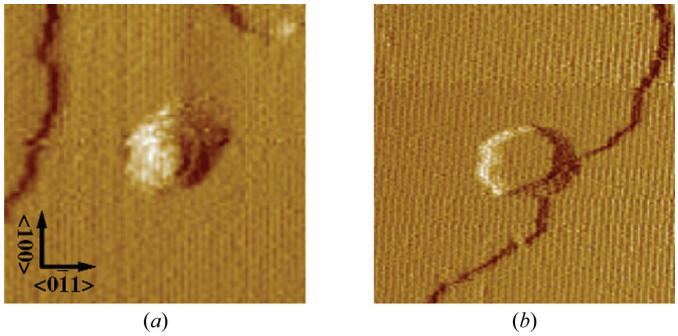


Figure 13
An example of normal growth is provided here by the surface of an actively growing apoferritin crystal. Nucleation is so rapid and unrestrained that growth in the face normal direction competes favorably with tangential growth. This mixture of growth modes leads to the observed rough and chaotic texture. The scan area is 25 × 25 μm.



(*a*) (*b*)

Figure 14
In (*a*), on the surface of a thaumatin crystal, a disordered three-dimensional nucleus appears. In (*b*) the nucleus has restructured into a two-dimensional island having crystalline order, and the island is properly oriented with the underlying lattice. During this transformation the step edge on the left in (*a*) has expanded tangentially underneath the island. Scan areas are 4 × 4 μm.

An intriguing question is the origin of these multilayer stacks. A possible explanation, for which there is some evidence, is that they arise from liquid protein phase droplets (Kuznetsov *et al.*, 1998; McPherson *et al.*, 2001) that exist in concentrated macromolecular solutions (Asherie *et al.*, 1996; Haas & Drenth, 1999; Kuznetsov *et al.*, 1998, 1999; Liu *et al.*, 1995; Rosenbaum *et al.*, 1996), particularly in mother liquors, where the concentration of precipitating agents (which promote association) may also be very high. The liquid protein phase droplets are thought to be composed of hundreds to thousands of molecules exhibiting short-range order mediated principally by nonspecific van der Waals interactions and random arrangements of hydrogen bonds. They are in a sense very large disordered protein aggregates, not unlike the condensed pools of virus particles seen in Fig. 4. Because of the extraordinary concentration of molecules in the droplets, they are locally hypersaturated. When the droplets sediment upon broad crystal surfaces, the preexisting lattice serves as an epitaxial substrate to guide and promote crystallization in the molecules above. Each new layer then inspires crystallinity in the molecules above them and so forth, propagating a continuous series of growth layers: an ordered multilayer stack. In some rare cases, they form independent microcrystals which are discontinuous with the underlying lattice (Fig. 16*c*).

The existence of a liquid protein phase in concentrated protein solutions, of which the multilayer stacks discussed here are one manifestation, has been dealt with in greater detail elsewhere and is the source of much current interest in the field of colloids as well as crystal growth (Asherie *et al.*, 1996; Kuznetsov *et al.*, 1999; Liu *et al.*, 1995; ten Wolde & Frenkel, 1997; Piazza, 1999). It was one of the more unexpected results to emerge from AFM studies of macromolecular crystal growth and it may have consequences for the physical chemistry and structure of concentrated macromolecular solutions. It could provide an explanation, or a pathway, not only for the mechanism of crystal growth through three-dimensional nucleation, but also for the spontaneous formation in solution of crystal nuclei having critical size (Asherie *et al.*, 1996; Haas & Drenth, 1999; Rosenbaum *et al.*, 1996; ten Wolde & Frenkel, 1997; Piazza, 1999).

A truly remarkable feature of crystal growth as visualized on the surfaces of macromolecular crystals, regardless of mechanism, is the seamless manner in which advancing step edges encounter one another and perfectly knit together (Fig. 17). There are few, if any, phenomena on this scale in nature that match this for precision. The merging of step edges from multiple two-dimensional islands and from dislocations on growing surfaces must occur millions of times in the course of crystal growth, and virtually without fault (for exceptions, see §8). This exactness can only be a consequence of the extraordinary molecular guidance provided by the underlying crystalline surface, *i.e.* by homoepitaxy.

It merits emphasis that protein crystals do not necessarily develop using exclusively one of the mechanisms described here, although they may do this as well. Not only might different crystal faces support different growth modes, but in some cases two or more mechanisms may be active on a single crystal face simultaneously. A mechanism that dominates at one supersaturation may give way to another as the degree of supersaturation is increased or decreased. Generally, growth by screw-dislocation generation of steps pertains

at very low supersaturation, and two-dimensional nucleation more generally, but this is joined by normal growth or growth through the appearance of three-dimensional nuclei at high supersaturation. This is illustrated by the growth of a tRNA crystal as the temperature is lowered, and hence as the supersaturation is increased, in Fig. 18. In passing, it might be noted that the experiment captured in Fig. 18 also demonstrates that crystals of nucleic acids grow by the same mechanisms as do protein and virus crystals.

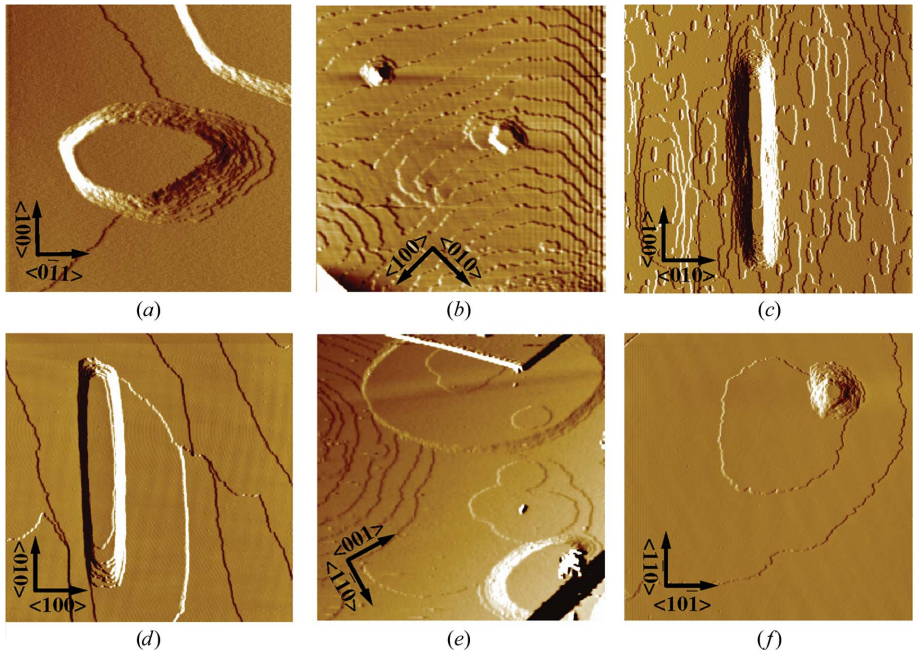


Figure 15
Multilayered stacks of growth layers arising from disordered three-dimensional nuclei often assume a morphology characteristic of a particular kind of crystal. Multilayer stacks are seen on the crystal surfaces of (*a*) thaumatin, (*b*) canavalin, (*c*) lipase, (*d*) catalase, (*e*) lysozyme and (*f*) STMV. The average height of the stacks is usually eight to ten growth layers, but stacks of only two growth layers and others of several hundred growth layers have also been observed. The shapes of the multilayer stacks depend on the anisotropic velocities of the growth steps at the edges of the two-dimensional islands. The scan areas are (*a*) 10 × 10 µm, (*b*) 25 × 25 µm, (*c*) 15 × 15 µm, (*d*) 25 × 25 µm, (*e*) 40 × 40 µm and (*f*) 15 × 15 µm.

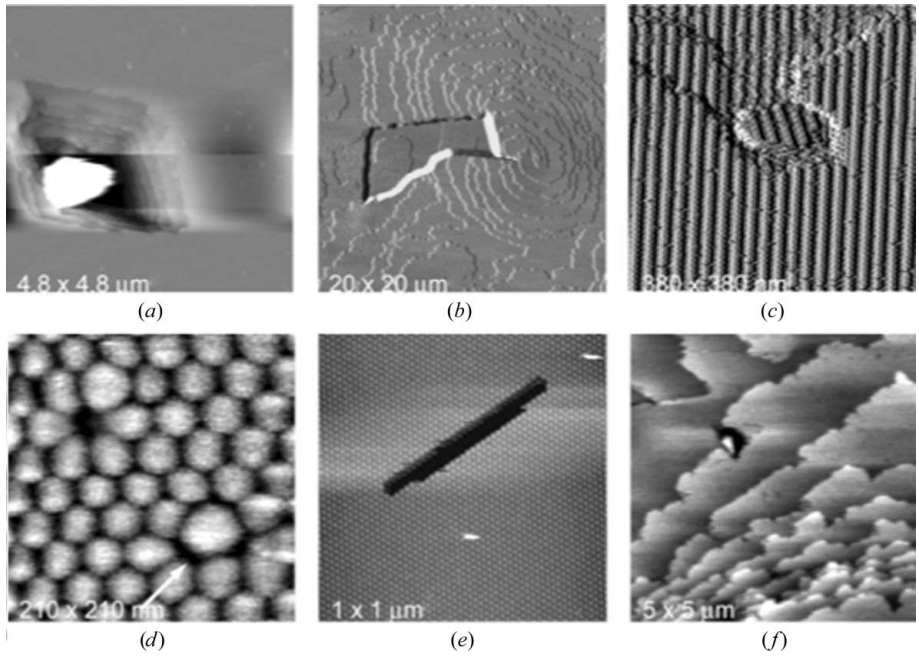


Figure 16
Impurities incorporated into macromolecular crystals come in a large variety. In (*a*) a dust particle within a lysozyme crystal is revealed by back etching. In (*b*) an independent microcrystal is wholly incorporated into a large, growing canavalin crystal. In (*c*) a thaumatin crystal subsumes a misoriented microcrystal. In (*d*) mutant virus particles of excessive size, one of which is denoted by an arrow, are nonetheless incorporated into the lattice of a *Brome mosaic virus crystal*, producing attendant local defects. In (*e*) a residual cytoskeletal fiber from a microbe leaves a distinctive scar after its consumption by a growing STMV crystal and in (*f*) the step edge on the surface of a growing canavalin crystal is seen rolling over an errant contaminating particle.

6. Thermodynamic and kinetic parameters

The interfacial free energy α of a growing crystal, its most fundamental thermodynamic parameter, can be directly determined by several means (Chernov, 1984; Chernov *et al.*, 1988; Rosenberger, 1979; Vekilov & Chernov, 2002). α provides a measure of the work required to create a unit of new surface area on a growing crystal, or alternatively, by normalizing to the entire surface area of a molecule, it quantifies the total amount of work required to remove molecules from solution and incorporate them into an expanding lattice. The value of α is important because it has implications for the relative chemical and physical environments and interactions that molecules enjoy in solution and in the crystal. From AFM studies (Malkin, Kuznetsov *et al.*, 1995; Land *et al.*, 1995) and from QELS analyses, which can also yield estimates of α (Malkin & McPherson, 1993*a*,*b*; Malkin *et al.*, 1993), the interfacial free energy was determined for ferritin, apoferritin, STMV and pumpkin seed globulin. For these macromolecular crystals, α was found to be roughly one to two orders of magnitude lower than for small molecules recruited from solution into a conventional crystal. When one considers the surface area of a macromolecule or virus, however, then the total energy required to incorporate it is about the same as for a small-molecule crystal (Chernov, 2003; Chernov & Komatsu, 1995; Vekilov & Chernov, 2002; Malkin & McPherson, 1994).

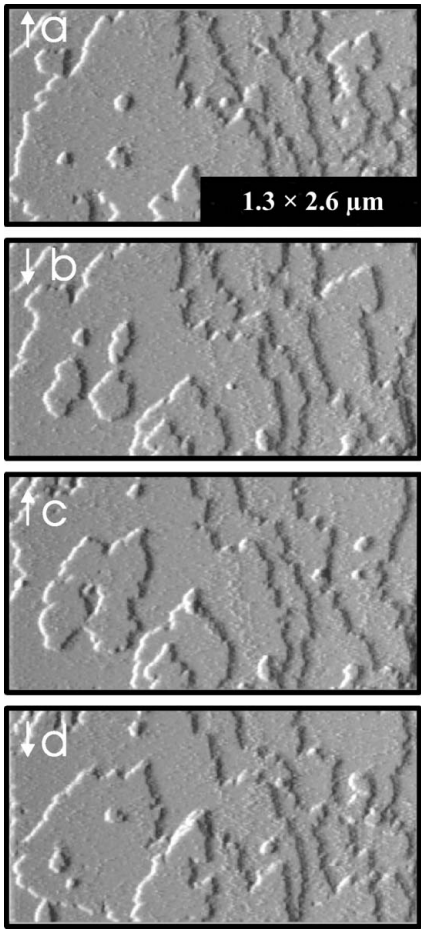


Figure 17
In this series of AFM images of the surface of a growing trypsin crystal, it can be seen that critical two-dimensional nuclei appear, give rise to two-dimensional islands that spread tangentially and finally merge to produce coherent islands. The arrows denote the rastering direction of the AFM scanner.

The low value of α associated with macromolecular crystals confirms, as might have been anticipated, that the environment of proteins, nucleic acids and viruses in their crystals is not, from an energetic standpoint, appreciably different than when they are free in solution. One might speculate further that this is so because the degree of hydration of macromolecules in solution is little changed upon entering a crystal, which is usually composed of about 50% solvent (Gilliland, 1988; Gilliland *et al.*, 1994; McPherson, 1999; McPherson, 1982). The structure of the water surrounding the macromolecule may, however, be changed.

The fundamental kinetic parameter governing crystal growth is the kinetic coefficient β (Chernov, 1984; Chernov & Komatsu, 1995; Rosenberger, 1979; Vekilov & Chernov, 2002). β is a measure of the kinetics of incorporation of a molecule into the crystal lattice owing to all processes: the combined kinetics of transport, absorption, surface diffusion, reorientation and any other components of the incorporation process, with the slowest of these being rate-limiting. Although estimates of β have been obtained for several macromolecules using AFM (Malkin, Kuznetsov *et al.*, 1995; Malkin *et al.*, 1999, 2001; see also Table 5 of Vekilov & Chernov, 2002), the most accurate approach is through the application of Michelson interferometry to growing crystal surfaces. This method, pioneered by Chernov and coworkers in Russia (Chernov, 1984; Chernov *et al.*, 1988), is based on the interference of a wavefront, reflected from a growing crystal face, with a reference beam. Phase shifts occur in the reflected beam with respect to reference because of height variations on the crystal surface owing to growth hillocks (Fig. 19) and two-

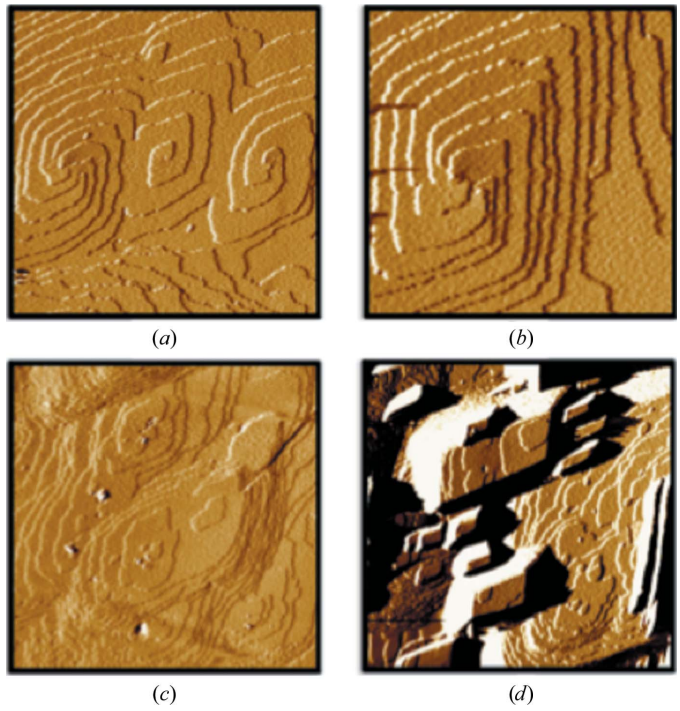


Figure 18
A decreasing temperature increases supersaturation and produces subsequent changes in the growth mechanism as seen here for an actively growing crystal of yeast phenylalanine tRNA. In (*a*), at a temperature of 15°C where supersaturation is low, growth is dominated by a variety of screw dislocations that create dislocation hillocks. In (*b*), at 14°C, the screw dislocations are degenerating and the step edges are roughening. In (*c*), at 13°C and higher supersaturation, two-dimensional nucleation dominates and screw dislocations have virtually disappeared as a source of new layers. In (*d*), at 12°C where the supersaturation is highest, two-dimensional islands are still present but three-dimensional nuclei and large macrosteps predominate. The scan areas are (*a*) and (*b*) 23 × 23 µm, (*c*) 20 × 20 µm and (*d*) 34 × 34 µm.

dimensional islands. Because, as with AFM, the measurements are carried out *in situ* in the mother liquor as the crystal grows, a continuous series of interferograms, such as those shown in Fig. 20, are obtained.

In the interferogram the distance between two successive dark (or two successive light) fringes corresponds to a height difference on the crystal surface of one wavelength of the laser light employed. The density of the fringes around any growth center is a quantitative measure of the height of the growth hillock at the center. The radial velocity of the fringes, measurable in time sequences, is related to the velocity of advancing step edges (Chernov *et al.*, 1988; Kuznetsov *et al.*, 1995; Vekilov *et al.*, 1992), and this is known as the tangential velocity. As noted above for AFM studies, from the tangential velocity one can also calculate α, the interfacial free energy.

If one were to choose a fixed point, at the growth center or otherwise, and then measure the period of change at that point from light to light or dark to dark, then the time required for one wavelength of growth perpendicular to the crystal surface is known. This is the face normal growth rate. From the tangential and normal growth rate, the kinetic coefficient β can be calculated. Indeed, it has been determined for the growth of crystals of the protein canavalin and crystals of *Turnip yellow mosaic virus*, the latter being a $T = 3$ virus of diameter 28 nm (Canady *et al.*, 1996). The values of β, consistent with other observations, were found to be roughly an order of magnitude lower than for conventional crystals.

7. The incorporation of three-dimensional nuclei, microcrystals and particles into crystals

Many prominent features of protein, nucleic acid and virus crystals and their development are associated with or ascribed to the incorporation of impurities into the crystal lattice. These include, among others, the kinetics of growth, restriction of ultimate size, limitations on diffraction resolution, habit or morphology, and the extent and structure of defects and dislocations. This might have been anticipated considering the extensive evidence from studies of conventional small-molecule and ionic crystals showing that impurities dramatically affect their degree of perfection (Hurle, 1994; Sarig, 1994; Rosenberger, 1979; McPherson *et al.*, 1996; Chernov *et al.*, 1988). Deleterious consequences have been demonstrated for many conventional crystals where impurities were present in only very small amounts, measured in parts per million or less, and even when extreme measures were taken to eliminate them from the mother liquor or melt from which the crystals were grown.

The situation is vastly more complicated in the case of macromolecular crystals (Malkin, Kuznetsov & McPherson, 1996*b*; McPherson, 1996). Proteins, nucleic acids and macromolecular complexes are, by their very nature, difficult to purify to homogeneity and to free from contaminant macromolecules. In biochemical studies, a protein that is 99% pure is considered exemplary, and levels of other individual proteins of less than 1% can in fact be detected using only the most stringent techniques such as silver-stained gels, isoelectric focusing or mass spectrometry.

Even highly purified macromolecules intended for crystal growth may, for a great variety of reasons, be chronically heterogeneous owing to post-translational modifications, denaturation, the binding of ligands or a myriad of other effects (McPherson, 1996, 1999; McPherson & Cudney, 2006). In addition, macromolecules are always crystallized from generally complicated solutions that may include not only the intended macromolecule, but also buffers, salts, precipitating agents, water or a wide range of possible effector molecules of conventional sizes. For additional details regarding mother-liquor components, see McPherson & Gavira (2014).

It is noteworthy that macromolecular crystals are composed not only of protein or nucleic acid, but also, as noted previously, a very large percentage of water (Gilliland, 1988; Gilliland *et al.*, 1994; McPherson, 1999). There are generally large interstices, cavities and channels within crystals filled with disordered solvent and its components. In some cases the sizes of the channels and cavities inside the crystals exceed even the dimensions of the macromolecules

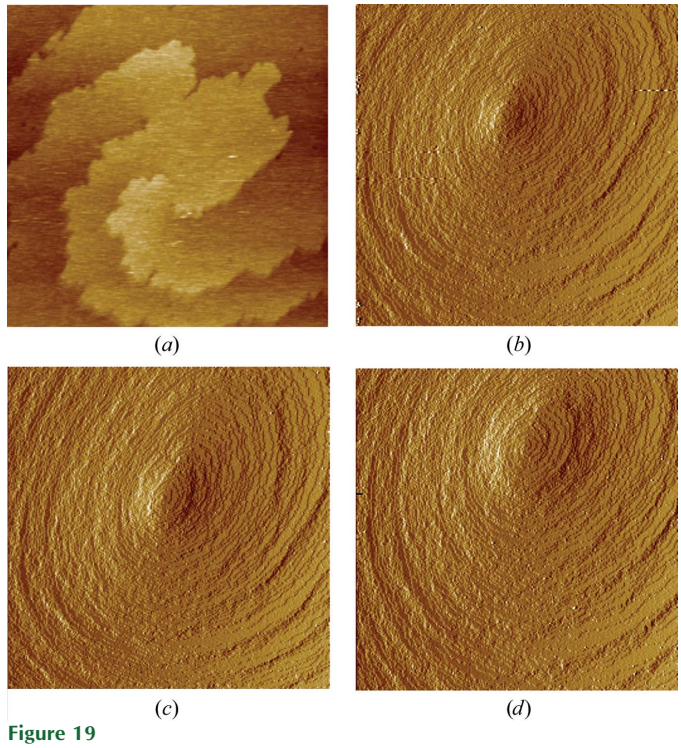


Figure 19
(*a*) The center of a screw dislocation on the surface of a trypsin crystal that gives rise to a dislocation hillock that sees continued development in (*b*)–(*d*). Scan areas are (*a*) 10 × 10 µm and (*b*–*d*) 25 × 25 µm.

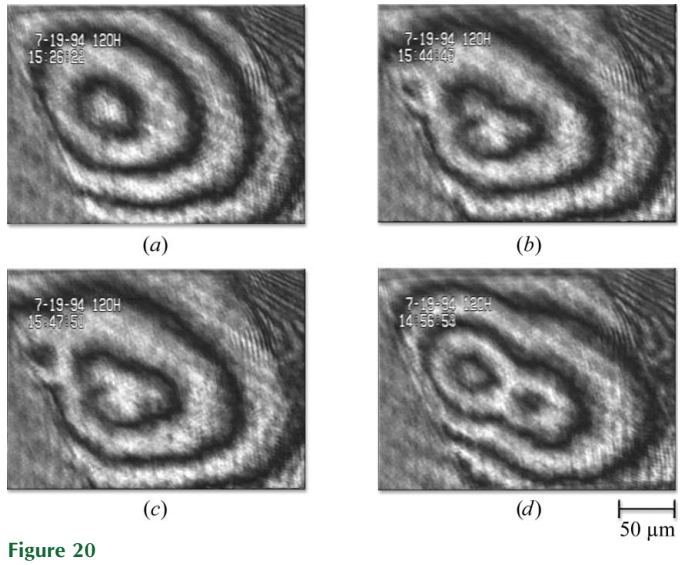


Figure 20
Michelson interferometric fringes that reflect the development of growth hillocks on the surface of a growing canavalin crystal. The fringes provide measures of the heights of the hillock and their rate of change measures the speed at which the hillocks develop in the face normal direction.

otherwise making up the crystals. Thus, molecules may exchange positions within the lattice or assume disordered orientations within a lumen. In addition, individual macromolecules themselves are surrounded in the lattice by shells of ordered, bound solvent molecules, which may vary in their affinities and positions (Frey, 1994).

Macromolecular crystals might therefore be expected to accumulate greater quantities, and a more diverse range, of impurities (Fig. 16) because of their unique character. On the other hand, up to a point at least, they often appear remarkably forgiving of the presence of impurities, even large ones, and their incorporation (Malkin, Kuznetsov & McPherson, 1996*a*; Malkin *et al.*, 1996*b*; McPherson, 1996). Ionic and small organic molecule crystals, by virtue of their strong intermolecular lattice interactions, tend to be brittle and therefore very sensitive to lattice disruptions arising from impurity incorporation. Macromolecular crystals, on the other hand, as a consequence of the mostly water-mediated lattice contacts, are generally plastic and therefore insensitive and accommodating. Macromolecular crystals can incorporate particles, fibers, other physical phases, microcrystals (Fig. 21), improperly oriented two-dimensional nuclei and a variety of small objects that approach a significant fraction of the ensemble in size. Frequently, the impurities are of such a size that they can be visualized using nothing more than a common light microscope (Fig. 22).

Occasionally, foreign particles or surfaces have served as nucleation centers (McPherson & Schlichta, 1988, 1989) and crystals simply grow around them. Particles, including other small crystals in arbitrary orientations, sometimes sediment upon or attach to growing crystals and are subsequently incorporated (Malkin, Kuznetsov & McPherson, 1996*b*; Malkin *et al.*, 1997; McPherson, 1996). This phenomenon also encompasses the inclusion of bubbles of gas, oil droplets and precipitated protein. A classic although qualitative indicator of the degree of incorporation of impurities is the roughness or irregularity of growth islands and step edges (Chernov, 1984). In the ideal case of no impurities, theory would predict step edges to be smooth at the molecular scale. For macromolecular crystals, however, this is seldom the case, and it is often far from it. Step edges for protein and virus crystals are ragged and coarse, as seen in Figs. 23 and 24. Such images provide ample evidence for the extremely impure nature of the samples with which we work.

Given a broad enough definition, one can also consider as impurities those portions of a macromolecule or molecular complex that are substantially disordered. Because of the imprecision of their orientation and position in the lattice, they can affect local relationships and produce effects similar to those resulting from other types of impurities: defects, dislocations and discontinuities. The complexity of biological structure provides a wealth of opportunities for such 'structural impurities'. Flexible or plastic macromolecules, particularly those having multiple domains such as immunoglobulins, have frequently been observed in X-ray studies to display some inherent disorder. Crystals of such molecules generally diffract less well than those of rigid, stable molecules. Observations of disorder in crystals and crystals that diffract to only low resolution are common with malleable membrane-protein crystals (DeLucas, 2009).

The inability to crystallize many flexible proteins or nucleic acids is also commonly ascribed to dynamic or statistical molecular disorder. Problems are magnified for glycoproteins, where oligosaccharide moieties usually display a spectrum of conformations. As with other mobile proteins, the quality of diffraction patterns are limited and significant difficulty accompanies their crystallization. Crystals of large macromolecular complexes represent even more challenging problems. Elements of the complexes may occasionally be missing, and multiple modes of association are possible. Virus crystals may contain both intact particles, empty capsids lacking nucleic acid and even mutant virions (Fig. 16*d*) of irregular sizes and morphology (Kuznetsov *et al.*, 2000; Malkin *et al.*, 2001).

Macromolecular crystals are almost invariably grown from highly concentrated solutions that encourage natural association of molecules to form both specific aggregates as well as random clusters. These too may enter into the crystal lattice and, indeed, there is

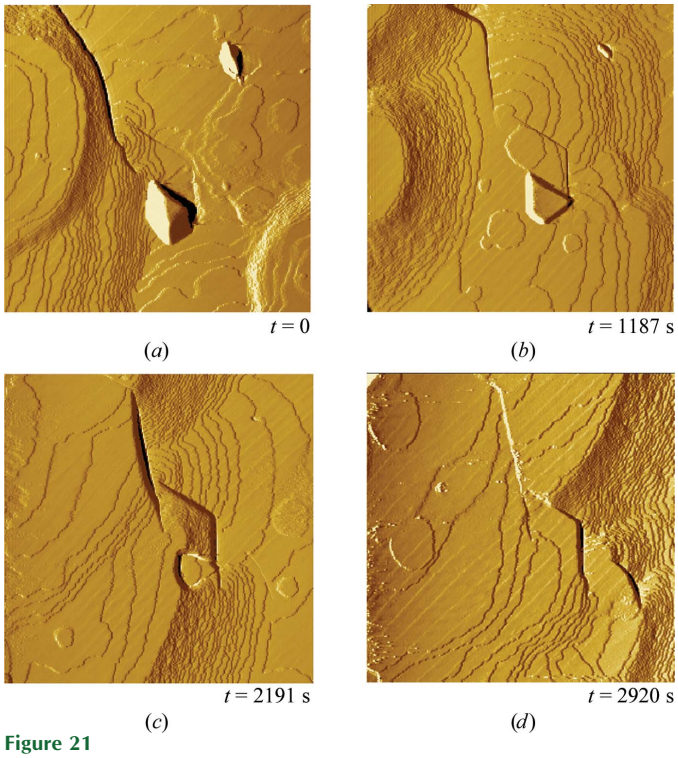


Figure 21
The phenomenon of microcrystal capture and incorporation is illustrated here by the sedimentation of two small crystals onto the surface of another much larger and rapidly growing STMV crystal. Steps advancing on the surface of the large crystal simply overwhelm and submerge the microcrystals. They thus become consumed and bodily incorporated into the bulk of the larger crystal. Scan areas are 25 × 25 μm.

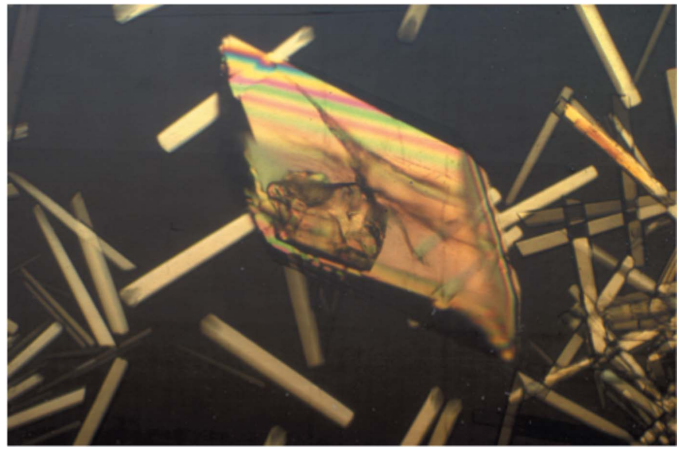


Figure 22
In experiments on heterogeneous and epitaxial nucleation of protein crystals on mineral surfaces (McPherson & Schlichta, 1989), a hexagonal canavalin crystal nucleated initially on the surface of a mineral particle of about 0.5 mm in size and then proceeded to grow to nearly 2 mm in length. In the process it completely included or internalized the mineral particle. Vast internal faults are, however, clearly visible within the large protein crystal as a result.

evidence from inelastic light-scattering experiments (Li *et al.*, 1999) and AFM studies (Malkin, Kuznetsov & McPherson, 1996*b*) that they do. Molecular aggregates or clusters can also introduce radically misoriented molecules into the lattice and create dislocations as serious as those produced by completely foreign matter. Denatured, proteolytically damaged and chemically altered nutrient molecules pose similar problems.

One of the most intriguing and unexpected observations made with AFM was the incorporation by growing protein and virus crystals of microcrystals that had sedimented on their exposed surfaces (McPherson *et al.*, 1996; Malkin, Kuznetsov & McPherson, 1996*b*). Presumably, the microcrystals had nucleated in the bulk mother liquor and, by gravity or convection, settled upon or were directed to the face of the larger active crystal. Examples of this phenomenon are seen in Figs. 16(*b*), 21 and 25. The microcrystals are incorporated intact into the larger crystals, as they can, in fact, be recovered by etching the larger crystal in an undersaturated solution (see below).

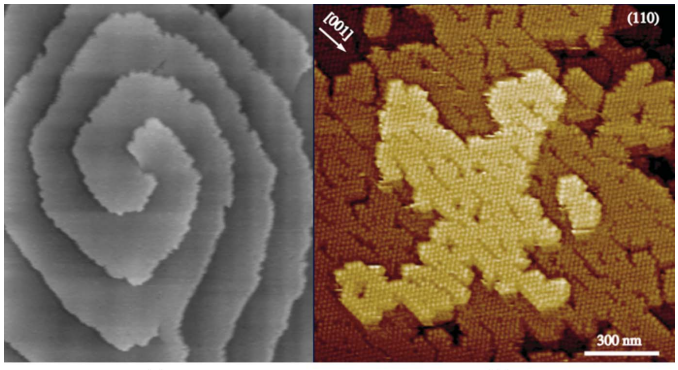


Figure 23
On the left is a double right-handed screw dislocation on the surface of a canavalin crystal, and on the right a dendritic two-dimensional island on the face of an orthorhombic STMV crystal. Note the roughness of the step edges in (*a*) and the irregular shape of the island in (*b*), both characteristic of extensive impurity incorporation. The scan area in (*a*) is 15 × 15 µm.

For some crystals, microcrystal capture is relatively unimportant and seldom occurs, but for others, such as catalase, STMV and canavalin, it may be common (McPherson *et al.*, 1996). The microcrystals are generally misoriented with respect to the lattice in which they become embedded, thus they contribute nothing to Bragg reflections. They do, however, contribute to the diffuse scatter and hence the background of diffraction intensities. Among other curious aspects, the incorporation of, in some cases, quite large (micrometre or larger) crystals serves to dramatically illustrate the sizes and varieties of impurities found in macromolecular crystals, and the resilience of their lattices.

It seems unlikely that macromolecular crystal growth, given the egregious offenses of impurities, can ever be carried out in a manner comparable, in terms of purity, to that currently attainable in the growth of conventional crystals. It is also unlikely, however, that the problems associated with each class of impurity simultaneously plague every macromolecular crystal-growth enterprise. More probably, some one impurity, or some small subset, will predominate in a specific instance, and the removal of these particular sources of impurity may serve to alleviate growth problems. In other cases, crystal growth may be influenced by a wide variety of impurities and minimization of the sum may be necessary. It cannot be emphasized strongly enough that greater purity can ameliorate a host of ailments.

8. Defects and dislocations in macromolecular crystals

It is clear that the levels of impurities and contaminants in macromolecular solutions, despite the greatest care, vastly exceed those in conventional crystal-growth solutions (McPherson *et al.*, 1996; McPherson, 1976, 1982, 1999). This is unavoidable, as it arises as a consequence of the inherent complexity of macromolecules, their sources and the accessory molecules and ions that may be needed to sustain them. Although there is no systematic evidence in support, intuitively we might conclude that the kinds of impurities that are most detrimental to macromolecular crystal growth are impurities in the size range of the nutrient protein molecules or larger. This seems

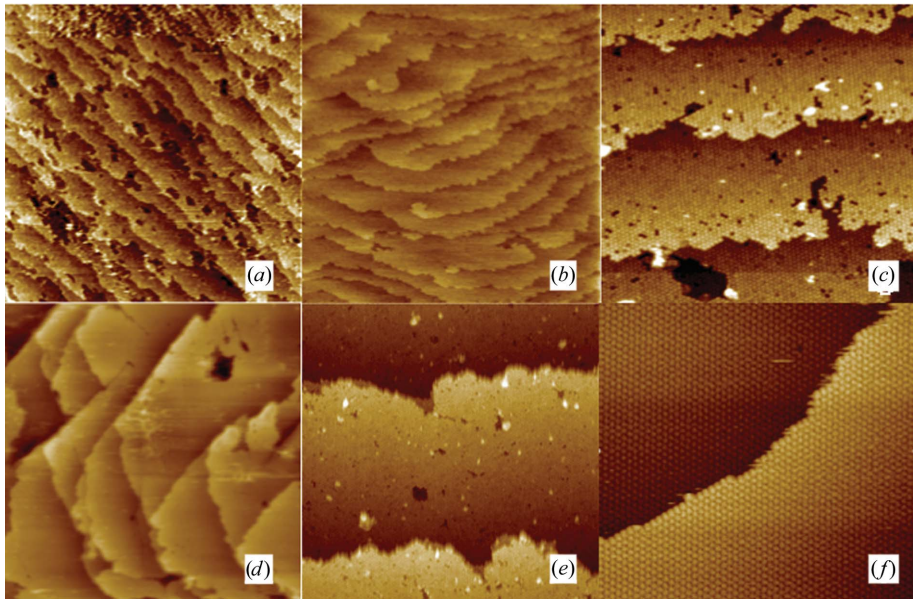


Figure 24
AFM images of advancing step edges on the faces of macromolecular crystals of (*a*) an intact monoclonal antibody against canine lymphoma, (*b*) canavalin, (*c*) and (*e*) STMV, (*d*) 50 s ribosomal subunits and (*f*) *Cucumber mosaic virus*. Note the extraordinary roughness of the step edges that are characteristic of high levels of impurity incorporation. Scan areas are (*a*) 5 × 5 µm, (*b*) 3 × 3 µm, (*c*) 2 × 2 µm, (*d*) 25 × 25 µm, (*e*) 3 × 3 µm and (*f*) 3 × 3 µm.

reasonable because these, if incorporated into a developing lattice, would be most likely to produce dislocations and deleterious defects. That is, the most damaging impurities to the crystal are likely to be misoriented, improperly folded proteins or molecules having alternative conformations. They would also include clusters or aggregates of the nutrient molecules, foreign particles such as dust, microcrystals and other contaminating macromolecules. We know from the discussion above that all of these types of impurities can, and do, become incorporated into crystal lattices.

The incorporation of impurities into the lattice, and the defects that they produce, may not be confined to their immediate neighborhood, but can have long-range effects on the overall structure of the crystal. In some cases only the molecules adjacent to the incorporated impurity may be jostled or perturbed by the elbows of their neighbor, but often not. Frequently, impurity incorporation is accompanied by inclusions or voids, or a resultant dislocation is propagated great distances through the lattice, affecting vast numbers of otherwise uninvolved molecules.

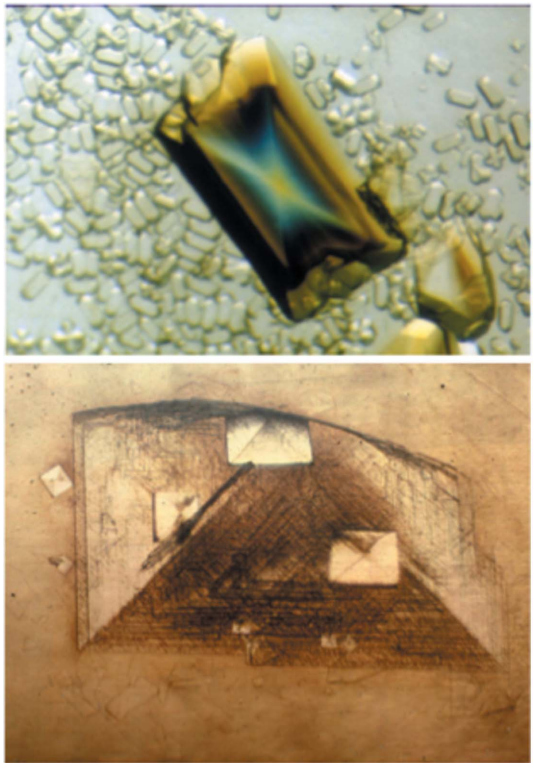


Figure 25
At the top is an orthorhombic crystal of STMV of about 1.5 mm in the longest dimension. It was grown in the International Microgravity Laboratory 1 in space. It is seen in its mother liquor of 15% saturated ammonium sulfate. Below is an STMV crystal that has been submerged in water so that it has experienced some dissolution. The etching reveals a host of otherwise invisible dislocations, defects, domains and their boundaries, and incorporated impurities, including microcrystals.

In the mother liquor in which a crystal grows, there are not only liquid protein-rich droplets and aggregates that can lead to three-dimensional nuclei and misoriented two-dimensional islands, but also spontaneously appearing microcrystals that can attach and be incorporated. This has been illustrated with particular clarity by investigations of merohedrally twinned (Ko *et al.*, 2001) rhombohedral canavalin crystals (Malkin, Kuznetsov & McPherson, 1996*b*; McPherson, 1999; McPherson *et al.*, 1996). Fig. 21 presents an example involving a crystal of the virus STMV. Microcrystals are drawn into the bulk of the larger growing crystal, and this is attended by the subsequent formation of defects.

Evidence for the extensive incorporation of impurities on the size scale of the nutrient molecules, perhaps representing misoriented individuals, clusters or other macromolecular impurities, is illustrated in Fig. 16. There, step edges generated by screw dislocations or two-dimensional islands on the surface of crystals are seen to be extraordinarily rough and ragged. The appearance is created by sites of impurity incorporation, called 'stoppers' (Chernov, 1984; Cabrera & Vermileya, 1958), that impede, in their immediate neighborhood, the progress of step edges.

Individual defects, and the overall defect structures (Cabrera & Vermileya, 1958; Chernov, 1984; Malkin, Kuznetsov & McPherson, 1996*a*; Tiller, 1991; Vekilov & Chernov, 2002), that are present in macromolecular crystals (Fig. 25) exhibit considerable variety. As an ensemble of faults, they give rise to crystalline domains and to the effect known to X-ray crystallographers as mosaicity. They also suggest why some crystals may appear less ordered and diffract to lower resolution than do others. A significant finding from AFM studies, which allow one to count defects and dislocations directly, is that some macromolecular crystals contain several orders of magnitude more dislocations than do most conventional crystals (Malkin, Kuznetsov & McPherson, 1996*b*; McPherson *et al.*, 1996).

Figs. 26, 27 and 28 present examples of some commonly observed defects in macromolecular crystals. Examination of the surfaces of virus and thaumatin crystals in Fig. 26 shows that they are strewn with absences corresponding to one or more unit cells. These are not filled later during growth, as etching experiments (see below) demonstrate. Vacant unit cells may account for as much as one to two percent of all of the cells in a crystal. Their effects, however, appear to be fairly localized and only a marginal diminution of order and quality results. Thaumatin crystals, for example, diffract to nearly 1 Å resolution. In Fig. 26, it is seen that crystals of viruses are similarly permeated by

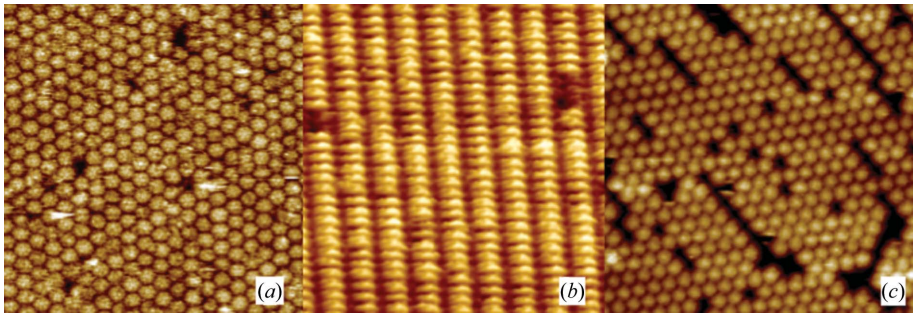


Figure 26
Examples of vacancies and line defects in macromolecular crystals. Crystals of (*a*) *Brome mosaic virus* (BMV), (*b*) thaumatin and (*c*) STMV are shown. The vacancies, or absences, are not filled as the crystal develops, but persist until crystal growth is complete. Scan areas are (*a*) 540 × 540 nm, (*b*) 225 × 225 nm and (*c*) 300 × 300 nm.

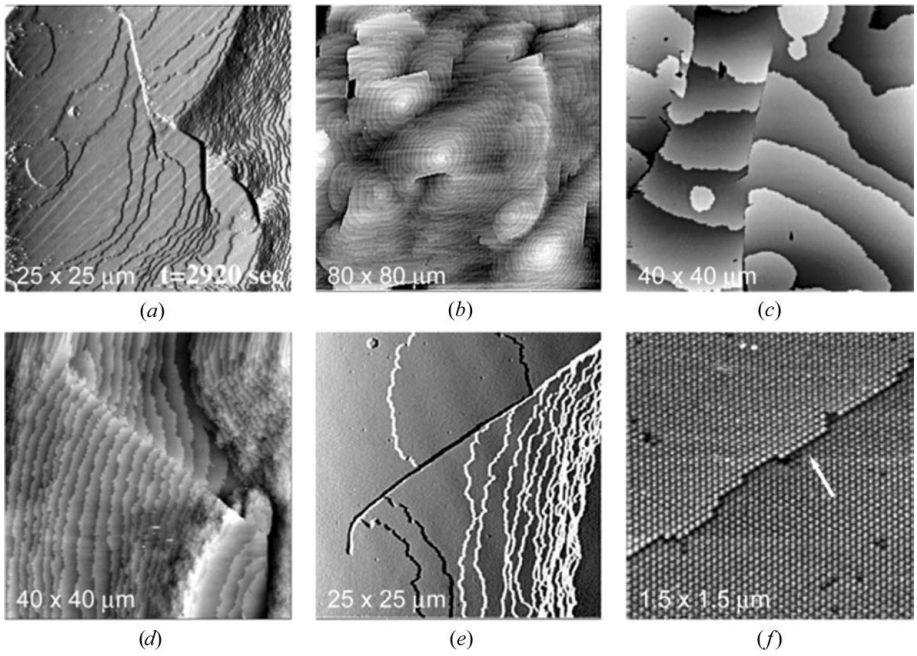


Figure 27
Planar defects (stacking faults) in crystals of proteins and viruses. Sufaces of crystals of (*a*), (*c*), (*d*) and (*e*) STMV, (*b*) canavalin and (*f*) CMV are shown. The planar defects, homologous to grain boundaries in conventional crystals, divide the crystal into domains, which in turn are responsible for the mosaicity of the crystals.

empty lattice sites. Like thaumatin crystals, the STMV crystals shown here diffract extremely well to nearly 1.4 Å resolution.

Defects of a considerably more serious nature are dislocations. These are harmful to long-range order because they extend great distances through the crystals. Screw dislocations have been discussed above. As noted there, screw dislocations may provide a useful source of new step edges required for face normal growth, particularly at low supersaturation. They do, however, extend along a line through the crystal and in the immediate neighborhood of the line dislocation the lattice must be disordered.

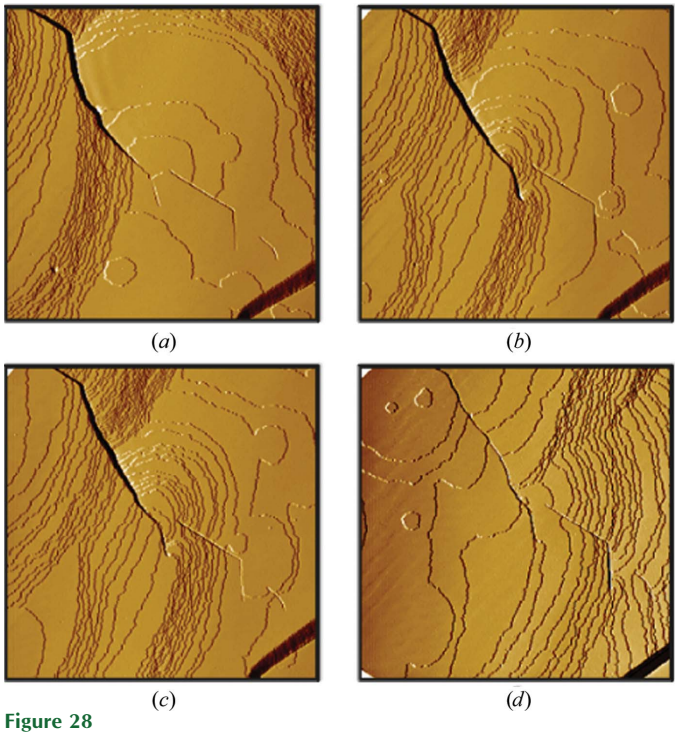


Figure 28
A series of AFM images showing the spread of layers and the disruption of their natural courses by a planar defect in an STMV crystal. Scan areas are 25 × 25 µm.

Another common defect is the stacking fault or planar dislocation. Fig. 27 provides examples of stacking faults on the surfaces of several different types of crystals, and Fig. 28 illustrates the disruptions and perturbations introduced by their appearance. Stacking faults arise from partial unit-cell displacements of an entire plane of unit cells perpendicular to the surface layer, often half a unit cell in height. When advancing steps encounter the faults they are unable to continue and must flow around the fault and up the other side. Thus, molecules affected by the fault may extend hundreds or even thousands of layers through the crystal. A block structure is thereby imposed on the crystal by the propagating displacements and these serve as the boundaries of domains in a crystal.

It is stacking faults or planar defects that are responsible for the mosaic character of crystals, including macromolecular crystals. One suspects that changes in the state of water within these faults when a crystal is cryocooled or flash-cooled may explain the general increase in mosaicity that is observed with crystals thus treated. The faults are similar to grain boundaries in conventional crystals in that they divide crystals into domains having slightly different dispositions with respect to one another. Each domain thus has a slightly different Bragg angle as a consequence, and this produces the spread in X-ray intensities. The sizes of the domains may be quite small as for some seen in crystals of *Cucumber mosaic virus* (Fig. 29) and the merohedrally twinned rhombohedral canavalin (Fig. 11), or they may be very large, as we find, for example, in STMV crystals.

Direct visualization of dislocation and defect distributions on a number of different crystals by AFM permitted measurement of the number of defects per unit area of the crystal surface (Malkin, Kuznetsov & McPherson, 1996*a*; McPherson *et al.*, 2000). For crystals of rhombohedral canavalin, which diffract to little better than 3 Å resolution, this was calculated to be between 10^5 and 10^6 defects per cm^3 (McPherson, 1999; McPherson *et al.*, 1996, 2000). If canavalin were representative, then protein crystals are dramatically more imperfect and have a substantially higher defect density than do most

conventional crystals. Canavalin, however, owing to its merohedral twinning (Ko *et al.*, 2001), is likely to have an exceptionally high defect density. Rhombohedral canavalin is a patchwork of domains with inverted orientations. Domain boundaries where discontinuities occur are therefore common, and these give rise to dislocations. Even for most macromolecular crystals, however, it is likely to be one to two orders of magnitude higher.

Molecules influenced by defects may contribute virtually nothing to Bragg reflections because of their misorientation (and can even detract), but as noted above do contribute to the diffuse scatter of the crystal and therefore the background intensity. We would therefore expect protein crystals with particularly high dislocation densities to exhibit low I/σ ratios over the entire resolution range, and in some, if not most cases to provide a diffraction pattern of rather limited resolution. It has not yet been shown definitively, but it seems a fair assumption that resolution and general diffraction quality are linked to crystal defect density.

Just as observations of crystal growth on the nanoscale are valuable in defining mechanism and kinetics, experiments on the dissolution of crystals can also be revealing. In particular, if dissolution can be made to occur in a slow and controlled manner, a process known as etching, then it becomes a useful approach for delineating the defect structure of a crystal and identifying the sites of impurity incorporation (Buckley, 1951; Monaco & Rosenberger, 1993; Malkin, Kuznetsov & McPherson, 1996*b*). Because of local strain imposed upon the lattice, the chemical potential is higher at defects and impurity sites than in ordered areas. As a consequence of lattice stress, dissolution, a loss of molecules from the crystals, occurs first at these higher energy sites. Point defects owing to absences, and the incorporation of misoriented or foreign molecules produce what are called etch pits. Etch pits not only mark the points at which impurities have been incorporated, but grow wider and deeper with time as the crystal proceeds to dissolve, and often allow visualization by AFM of the offending impurity. Etching provides a record of the errors committed and contaminants absorbed during the growth process

Etching reveals not only particles and point defects, but also microcrystals that were incorporated. Etching of crystals of bovine liver catalase, for example, produced a remarkable record of microcrystal incorporation by larger crystals, and this largely explained the poor diffraction quality of catalase crystals (Malkin *et al.*, 1997). Rectangular microcrystals, some more than a micrometre in length and having a variety of orientations inconsistent with the underlying lattice, were strewn throughout the crystals.

The forgiving nature of some larger crystals to microcrystal incorporation is impressive and surprising. Microcrystal incorporation into catalase crystals, for example, does not seem to produce either screw dislocations or stacking faults. Presumably, crystals have varying degrees of tolerance for microcrystal and other contaminant incorporation, and exhibit different mechanical properties, some accommodating it without undue stress and others not. The ability of macromolecular crystals to absorb such a variety of impurity types and sizes is remarkable. A corresponding phenomenon is not known for more brittle conventional crystals where lattice stress introduced by impurities, and their attendant defects, would in all likelihood abort development completely, or at least lead to splintering and fracture.

9. Termination of growth

A question that has vexed protein crystallographers for years is why protein crystals seem to reach a more or less constant terminal size and then cease growing further. This is observed even when measurements demonstrate that there is sufficient protein remaining in the mother liquor to maintain supersaturation. This phenomenon is known as 'growth termination'. Termination seems not to trouble the growers of crystals of conventional compounds. It appears that most small-molecule crystals can experience virtually unlimited growth as long as supersaturation is maintained and impurities are limited. The causes for growth termination in protein crystals appear to be twofold, although one it appears is more serious than the other.

Firstly, but probably less important, the level of supersaturation required for face normal growth (Fig. 1), *i.e.* the nucleation of new layers on a crystal face, is higher, and may be much higher, than that needed to support tangential growth. When crystallization has proceeded extensively and the concentration of protein in the mother liquor is correspondingly reduced, supersaturation may decline to a point where it can sustain tangential growth but is no longer adequate to promote two-dimensional nucleation. In the absence of screw dislocations, growth will cease.

A more serious problem, however, is the accumulation of impurities on developing crystal surfaces. As growth proceeds, it does so at a slower and slower rate as supersaturation declines. Slower growth in turn means that terraces on crystal surfaces are exposed for longer periods, which allows a greater build-up of impurities on successive layers (Chernov, 1984). Eventually, the impurity density on layers so impedes step-edge movement that advancement ceases.

Impurity layers, in the case of macromolecular crystals, appear to be particularly toxic. The impurities, we presume, are primarily denatured protein, protein aggregates and foreign proteins, all of which have some degree of chemical activity. That is, they can undergo photo cross-linking and a host of other types of covalent cross-linking, particularly when denatured. AFM experiments (McPherson *et al.*, 2001) have shown two important features of these impurity layers: firstly, that they constitute dense shells of relatively

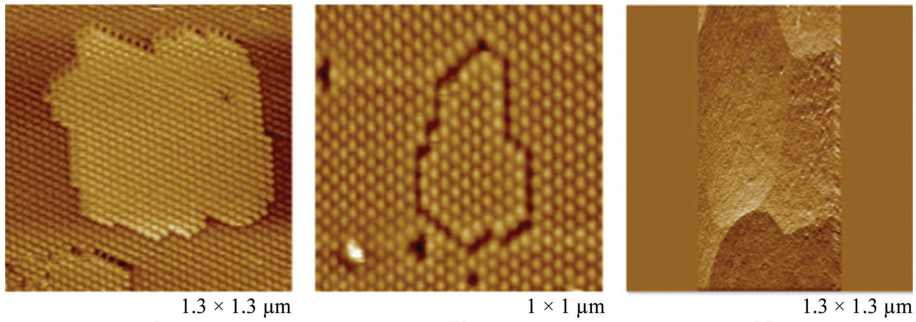


Figure 29
Domains and their boundaries created by planar defects in crystals of (*a*) and (*b*) *Cucumber mosaic virus* and (*c*) fungal lipase.

Figure 30
The background in these AFM images is representative of the 'hard shell' coating that ultimately forms on the surfaces of protein crystals, in this case lysozyme crystals, and produces growth termination. The shells are presumably composed of impurity molecules, aggregates and denatured protein, probably toughened by naturally occurring photo and chemical cross-linking. If the AFM tip is used to scrape the coating away, thereby exposing a fresh surface below, then growth resumes. This is demonstrated by the appearance of new step edges and two-dimensional islands on the newly presented surface. Scan areas are (*a*) 8 × 8 μm and (*b*) 7.5 × 7.5 μm.

hard material on crystal surfaces that are difficult to penetrate, and that prevent access to the interiors of the crystals and, secondly, that if the hard shell material is scrapped away to reveal fresh crystal below, then growth can again be seen to commence on the newly exposed surface. This is evident by the appearance of growth steps and two-dimensional islands on the fresh surface (Fig. 30). Some intriguing experiments have been carried out (Plomp *et al.*, 2003) using this approach to express fresh surfaces on 'dead' protein crystals, and it has been shown that it could potentially be a practical method for 'reviving' a dormant crystal.

10. A model for the growth of macromolecular crystals

To understand why the recruitment of a molecule, ostensibly free in solution, into a growing crystal is so slow for macromolecules, it is necessary to have a model that identifies and integrates the obstacles to incorporation that need to be overcome. A simple model for a molecule suddenly colliding with a surface and popping into place like an egg into a carton simply will not do. Models have been proposed that reflect a more realistic perspective on what is an intricate scheme of events; one that explains molecular capture and incorporation (McPherson, 1999; Vekilov & Chernov, 2002).

In terms of transport processes, molecules must (1) diffuse from the bulk solvent to a surface layer, where the properties of the fluid may be quite different. In this layer, not only will the local macromolecular concentration be lower, but the number of impurities, structure of the solvent, dielectric and likely other physical properties may be different as well. Molecules must then (2) diffuse through this surface layer, where they may experience turbulence owing to convection, and (3) encounter and adsorb to the crystal surface. Once adsorbed to the crystal, the molecule must (4) move by two-dimensional diffusion over the surface until it reaches a potential incorporation site while constantly experiencing the possibility of dissociation from the surface.

Once the molecule has reached the incorporation site, it must (5) undergo rotational diffusion until it achieves an orientation that allows it to form unstable, fluid bonds with molecules already present in the lattice. During or following these translational and rotational processes, the molecule may (6) have to undergo partial dehydration or rearrangement of its waters of hydration and possibly (7) undergo a conformational change to make it conform to the lattice building units.

Following this sequence, the molecule may have to (8) form some kind of quasi-stable but still fluid bonding to molecules in the lattice, perhaps involving van der Waals or transient hydrogen bonds. These bonds may then (9) undergo rearrangement to form the final crystalline binding arrangement. This may be accompanied, in addition, by (10) the liberation of additional water molecules. Finally, the stable incorporation of a molecule into a kink or lattice site is likely to require (11) the establishment of a water network between the new recruit and the previous members of the ensemble, as well as with the local water environment. Furthermore, one cannot ignore the possibility that while new molecules are being added to the crystal, old members may (12) be dissociating from the step edge. A low value for β indicates that the sum of these processes is indeed slow compared with conventional crystals, but it does not indicate which predominates, which is the rate-limiting step or if, in fact, any one step is dominant.

Many of the events described in this pathway may not exist or be so rapid as to be irrelevant from a kinetic standpoint. Nonetheless, it is useful to include as many possibilities as is reasonable if one is to fairly predict the effects of supersaturation, temperature, impurity type, impurity concentration, step-edge density or mechanism of step formation.

The problems encountered by a molecule attempting to join a growing lattice may be balanced to some extent by factors that facilitate the process. These, however, are uncertain and little more than hypothesis. Because the molecules are polyions, they exhibit a complex electrostatic surface. Even at their isoelectric point, the distribution is non-uniform, with patches of negative and positive charge scattered over the molecular surface. Macromolecules, by virtue of this charge distribution, are surrounded by a complex electrostatic field, and may exhibit an electrical dipole or quadrapole moment. Thus, they could, by Coulombic forces, be guided in their approach to a crystal surface and directed into preferred orientations. This could occur through interaction with a field established by the molecules making up the face of a crystal having complementary electrostatic properties. Thus, there could be a 'steering' effect that would lessen the dependence on 'pure chance' collisions and random orientation. Because of the transient nature of the hydrogen bonds between water molecules and a macromolecule, exchange during entry may be relatively simple. Disassociation of molecules from the crystal, at reasonable levels of supersaturation, can probably be ignored.

Our understanding of macromolecular crystallization remains, of course, unfinished. Like any science, it is never complete. The model presented above immediately suggests remaining questions, but there are undoubtedly even more. We are still unsure about the energetics involved in molecule incorporation, *i.e.* the enthalpic and entropic contributions to the process, their physical sources and the role that is played by the solvent (Israelachvili, 1995; Eisenberg & Kauzmann, 1969; Yau & Vekilov, 2000; Vekilov & Chernov, 2002). We are still unclear about the nucleation pathway and the properties of the aggregates and phases that exist in concentrated protein solutions. And, needless to say, we still cannot predict with any assurance what solution conditions will favor crystals over other phases and states. These and others remain as questions for future investigations, and most probably they also await the development and application of new technologies.

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