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Authors

Malkin, Alexander J
Kuznetsov, Yurii G
Lucas, Robert W
et al.

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Surface Processes in the Crystallization of Turnip Yellow Mosaic Virus Visualized by Atomic Force Microscopy

Alexander J. Malkin,[1] Yurii G. Kuznetsov, Robert W. Lucas, and Alexander McPherson

Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92697-3900

In situ **atomic force microscopy (AFM) was used to investigate surface evolution during the growth of single crystals of turnip yellow mosaic virus (TYMV). Growth of the (101) face of TYMV crystals proceeded by two-dimensional nucleation. The molecular structure of the step edges and adsorption of individual virus particles and their aggregates on the crystalline surface were recorded. The surfaces of individual virions within crystals were visualized and seen to be quite distinctive with the hexameric and pentameric capsomers of the *T* = 3 capsids being clearly resolved. This, so far as we are aware, is the first direct visualization of the capsomere structure of a virus by AFM. In the course of recording the *in situ* development of the crystals, a profound restructuring of the surface arrangement was observed. This transformation was highly cooperative in nature, but the transitions were unambiguous and readily explicable in terms of an organized loss of classes of virus particles from specific lattice positions. In some cases areas of a single crystal surface were recorded in which were captured successive phases of the transition. We believe this provides the first visual record of a cooperative restructuring of the surface of a supramolecular crystal.** © 1999 **Academic Press**

Key Words: **AFM; crystal growth; surface reconstruction; two-dimensional nucleation; virus.**

INTRODUCTION

Recent years have seen the convergence of a variety of technologies for the determination of the structural and even dynamic properties of supramolecular assemblies (King and Chiu, 1997; Steven and Spear, 1997). For example, cryo-electron microscopy

is now used as a means of obtaining low-resolution phase information for complex assemblies such as large viruses (Baker and Johnson, 1997), which is then extended to high resolution by X-ray diffraction. As we show here, atomic force microscopy (AFM) may also be a useful tool for obtaining similar information. Because it can be applied under essentially physiological conditions, in aqueous medium, it may in many cases be superior to other microscopic techniques that require dehydration, freezing, or staining or potentially otherwise perturb the structures of the samples in some way.

The clarity with which structural detail can be seen on the surfaces of small viruses, here turnip yellow mosaic virus (TYMV), which has a diameter of only about 28 nm, suggests that AFM may be even more broadly useful as an analytical tool. Many viruses cannot be crystallized at all or have unit cells beyond the range of X-ray crystallography. In those cases, as suggested here, AFM may provide an insightful approach to study not only large virus structure but also dynamic processes such as assembly and decapsidation (Casjens, 1985; Kaper, 1975).

In the past several years scanning tunneling microscopy (STM) has been successfully applied to studies of atomic mobility and surface evolution of crystalline materials grown in ultrahigh vacuum (Lagally, 1993; Zhang and Lagally, 1997). These results have been used in a number of practical applications, including development of electronic, optoelectronic, and superconducting devices. In contrast, experimental data on molecular dynamics on surfaces of macromolecular crystals, a governing factor in the development of a quantitative understanding of the crystal growth process, are virtually absent.

Scanning probe microscopies, including AFM, have also had a major impact on the field of nanoscale materials science, where they have provided information about molecular interactions and conformation (Jung *et al.,* 1997). They have also served as mechanisms for molecular repositioning (Jung *et al.,* 1996;

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[1] To whom correspondence should be addressed at Department of Molecular Biology and Biochemistry, University of California, Irvine, Room 560, Steinhaus Hall, Irvine, CA 92697-3900. Fax: (949) 824-1954. E-mail: amalkin@uci.edu.

Meyer *et al.,* 1997) and selective cleavage of molecular bonds (Gimzewski *et al.,* 1997). They have been used to investigate reconstruction of semiconductor surfaces upon cleavage, epitaxial growth, annealing, deposition of surfactant layers, and even controlled manipulation of individual atoms (Srivastava, 1997). These kinds of investigations have not been widely applied in structural biology because of the fragile character of the materials and their liquid environments.

This study represents an initial attempt to study structural features of macromolecules and molecular dynamics on surfaces of macromolecular crystals using a large particle amenable to both manipulation and detailed visualization. Using *in situ* AFM we have learned that the growth of the (101) face of TYMV crystals proceeds by two-dimensional nucleation, and we have been able to image growth step edges as well as visualize the adsorption of individual virus particles and their clusters on crystalline surfaces. The capsomere structures of virions immobilized within crystals of TYMV were visualized. A cooperative restructuring of the crystal surface was also observed.

MATERIALS AND METHODS

TYMV, a $T = 3$ icosahedral plant virus of 28 nm diameter, seen in Fig. 1a, is one of the most thoroughly studied of all viruses (Markham and Smith, 1949; Matthews, 1991; Rubio-Huertos *et al.;* 1967; Finch and Klug, 1966; Hirth and Givord, 1988; Mellema and Amos, 1972). The TYMV genome is made up of an $M_r = 1.9 \times 10^6$, single-stranded RNA of 6218 bases (Hirth and Givord, 1988) and a 694-nucleotide subgenomic RNA that is included in most virions. The structure of the virus, shown in Fig. 1a, was determined by X-ray diffraction analysis (Canady *et al.,* 1995,1996) from bipyramidal crystals (space group $P6_422$ with $a = b = 515.0$ Å and $c = 309.4$ Å) of TYMV, seen in Fig. 1b, grown from virus purified from Chinese cabbage by conventional procedures (Markham and Smith, 1949; Canady *et al.,* 1995). There are three virus particles in the unit cell and they are centered at $\langle\frac{1}{2}, 0, \frac{1}{2}\rangle$, $\langle 0, \frac{1}{2}, \frac{5}{6}\rangle$, and $\langle\frac{1}{2}, \frac{1}{2}, \frac{1}{6}\rangle$. The capsid of TYMV is composed of 180 identical protein subunits, each of about 20 kDa, organized into 12 pentameric and 20 hexameric capsomers that project about 40 Å above the surface of the virion (Mellema and Amos, 1972; Canady *et al.,* 1996).

TYMV crystals, seen in Fig. 1b, were nucleated on substrates in droplets of volume 10 μl by the vapor diffusion method (McPherson, 1998) consisting of mixing 10–16 mg/ml TYMV in H_2O with 0.8 to 1.0 M ammonium phosphate in 100 mM MES at pH 3.5. Crystals were then transferred into the AFM fluid cell, which was subsequently filled with a mixture of virus and precipitant solution. Images were collected in tapping mode using a Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) with Digital Instruments oxide sharpened silicon nitride tips. All operations were carried out under crystallization conditions, in a fluid-filled cell, with the supersaturation controlled by the concentration of precipitant, ammonium phosphate, or virus.

RESULTS AND DISCUSSION

We have studied crystals of TYMV, both their structure and their growth, by a variety of physical techniques that include Michelson interferometry (Kuznetsov *et al.,* 1995), X-ray diffraction (Canady *et al.,* 1995, 1996), and contact mode AFM (Malkin *et al.,* 1995a, 1995b, 1996a, 1996b, 1997). In earlier AFM investigations, however, we failed to observe growth steps or other recognizable features on the surfaces of TYMV crystals because of their exceptionally fragile character. The tapping mode method, however, has proven considerably more successful.

Under supersaturation conditions where crystal growth was clearly evident, high densities of growth steps were consistently observed on the (101) faces of TYMV crystals as illustrated in Figs. 2a and 2b. Growth occurred exclusively through two-dimensional nucleation and layer-by-layer step advancement. No dislocation sources were observed. Two-dimensional islands exhibited triangular shapes, as seen in Fig. 2b, which indicates kinetic anisotropy in step advancement along different crystallographic directions. The heights of growth steps (Fig. 2) were 29 ± 2 nm, which corresponds well with the (101) interplanar distances (30.6 nm) deduced by X-ray diffraction (Canady *et al.,* 1996). Step advancement proceeds through one-dimensional nucleation at the edges (Voronkov, 1970; Chernov, 1998; Rashkovich *et al.,* 1998, Kuznetsov *et al.,* 1999, in press, which results in kink formation and, subsequently, their lateral advancement.

Using tapping mode, not only were structures of step edges and their movements visible, but individual virus particles and their aggregates, adsorbed to crystalline surfaces, could be observed as well. In Fig. 3a, for example, an individual virus particle is seen adsorbed to the crystalline surface where it remained for an extended period of time. Figure 3b contains a cluster of nine virus particles on the crystalline surface. Over several scans the size of this cluster remained unchanged. After 10 min of observation (Fig. 3c), addition of a single new particle to the cluster was recorded as well as adsorption of several other individual virus particles to the surface. The experiments presented in Fig. 3 were conducted under supersaturation conditions close to equilibrium. At higher supersaturations much increased densities of adsorbed virus particles, as well as their clusters, were apparent on the crystalline surfaces. Detailed studies of molecular dynamics on the surfaces of TYMV crystals as a function of supersaturation, as well as underlying mechanisms of step motion, are currently under investigation and will be presented elsewhere.

Figure 4 presents AFM images of individual virus particles making up the (101) plane of the hexagonal crystals. Apparent in the images are broad, solvent-filled channels that permeate the crystals and which have diameters roughly equivalent to that of a
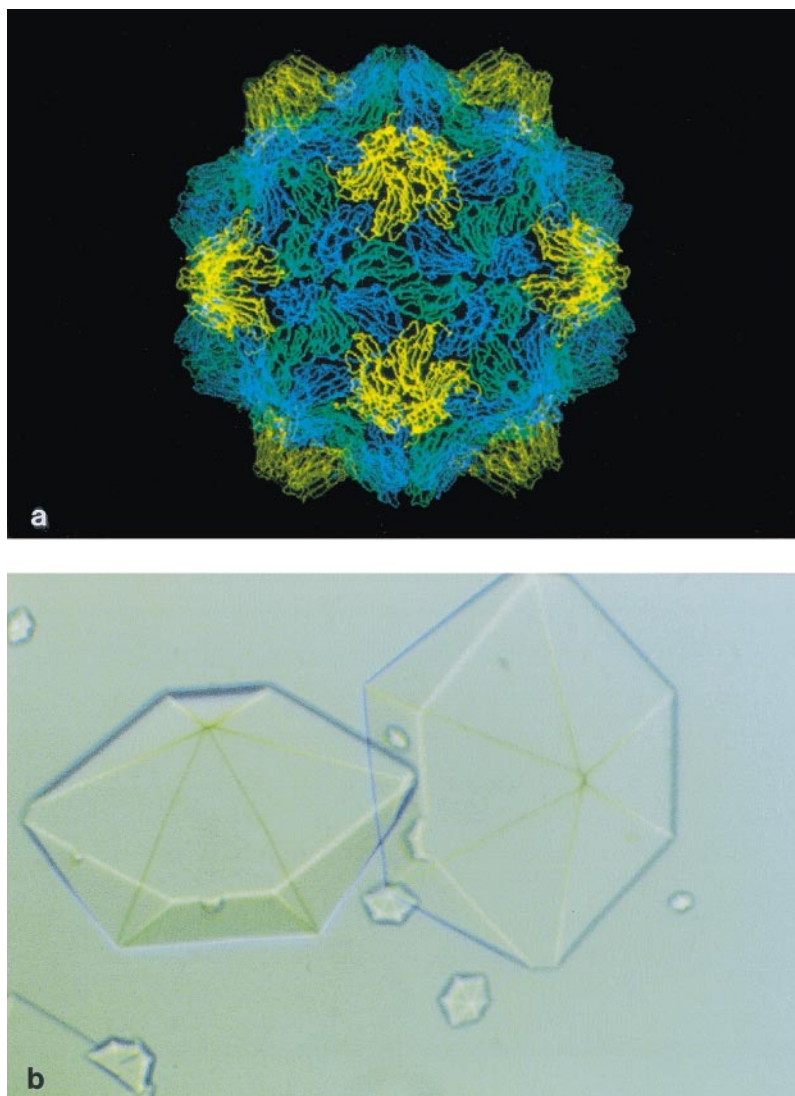
FIG. 1. (a) The structure of the capsid of TYMV based on X-ray diffraction analysis (Canady *et al.,* 1996) of the crystals shown in (b). The A subunits, which form pentameric capsomeres, are yellow. B and C subunits, which make up the quasi-hexameric capsomeres, are in blue and green. (b) Bipyramidal crystals of TYMV about 1 mm across.

virion. The images are reminiscent of those obtained of individual virus particles by negative staining electron microscopy (Finch and Klug, 1966; Mellema and Amos, 1972). The noteworthy features of the particles, which in this view are in the canonical orientation of the virion seen in Fig. 1a, are the capsomeres. Twelve of these are composed of five protein subunits, and 20 are composed of six protein subunits. Based on the known structure of TYMV and its arrangement in the crystals from X-ray diffraction data, as well as the 20% difference in their sizes, the pentameric and hexameric clusters can be discriminated from one another in Fig. 4. Note that the difference between the highest and the lowest points on the capsid surface, about 40 Å (Canady *et al.,* 1996), is accurately reflected by AFM.

We would emphasize again that the images of single virus particles provided by AFM, the accurate depiction of their structural characteristics, and their exact positions and orientations in the crystal lattice may prove useful for deducing initial phase information for X-ray diffraction. Initial phase information in virus crystallography is of particular value because structure determination relies only on extension of initial phases to high resolution using the icosahedral symmetry of the particles. The success of that process is directly dependent on the quality of the starting model used to formulate the initial phase set. The fact that capsomere structure is quite clear even on relatively small viruses such as TYMV is a propitious sign for the application of AFM to even larger, more complex samples.
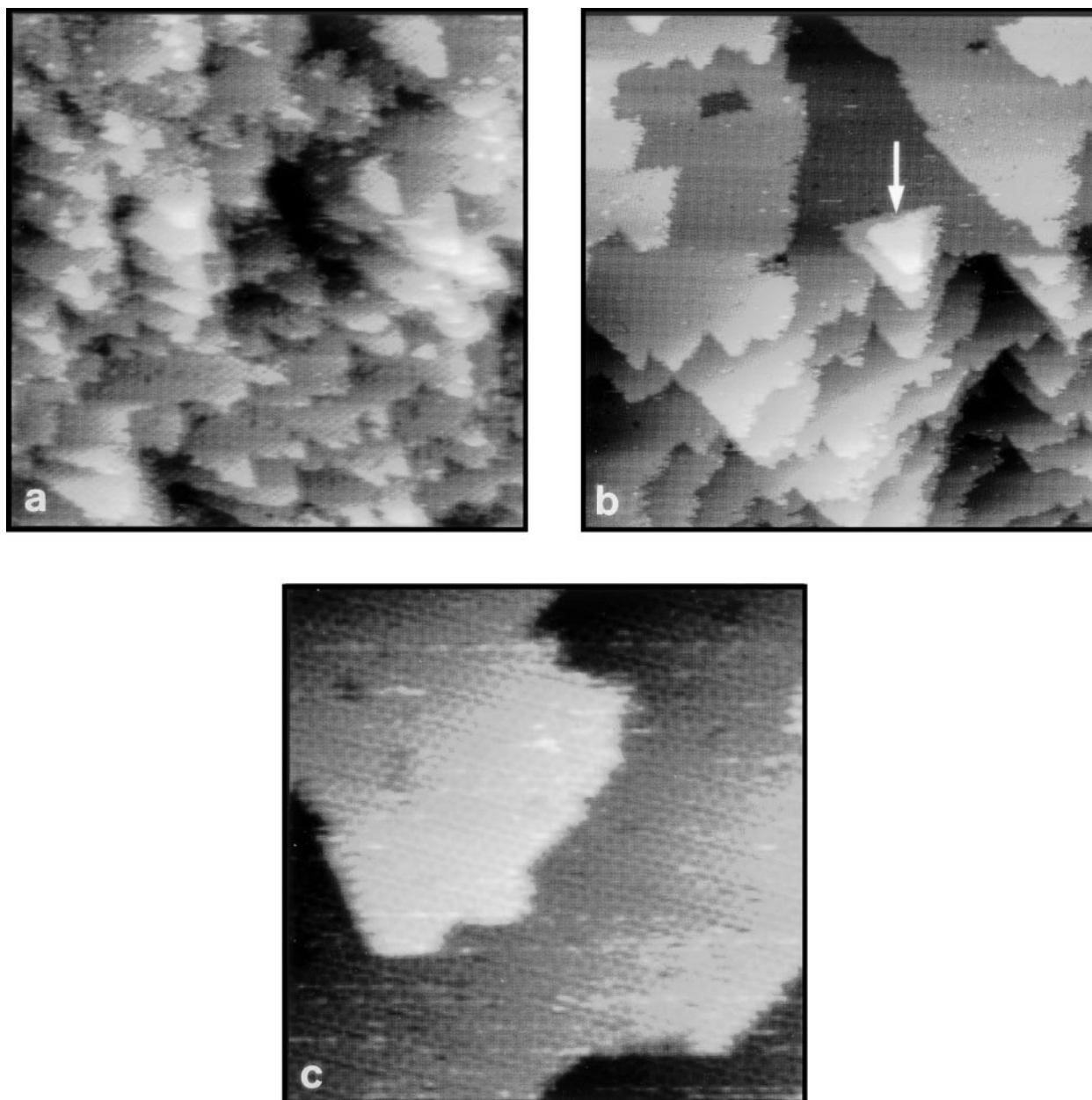
FIG. 2.   (a and b) Surface morphology of the (101) face of TYMV crystals. In (b) two-dimensional islands having a triangular shape are indicated with an arrow. (c) Structure of the growth step edge on a TYMV crystal surface. The scan areas are (a) 7×7 µm²; (b) 4×4 µm²; (c) 2.25×2.25 µm².

Larger scan areas of the surface layers of the (101) planes of the crystals revealed three distinctive patterns or arrangements of viral particles on the surfaces and these are presented in Fig. 5. When the supersaturation of ambient conditions was reduced to equilibrium, it was observed on a number of TYMV crystals that the patterns transformed in time from one to another. Transitions occurred rapidly, as one motif was seen at the end of a scan and another motif at the beginning of the next, where a scan period was about 4–6 min. Under these super-

saturation conditions no two-dimensional nucleation was observed and the step growth rate was relatively low. There were no steps sweeping across the surface areas under observation during these transitions. In some images, contiguous areas of the surface lattice exhibit sequential patterns, as in Fig. 5d.

A defined area on the crystal surface yields a consistent, stable pattern over many consecutive scans before the restructuring occurs; hence we believe that the tip does not displace individual, weakly bound particles from the lattice. We cannot,
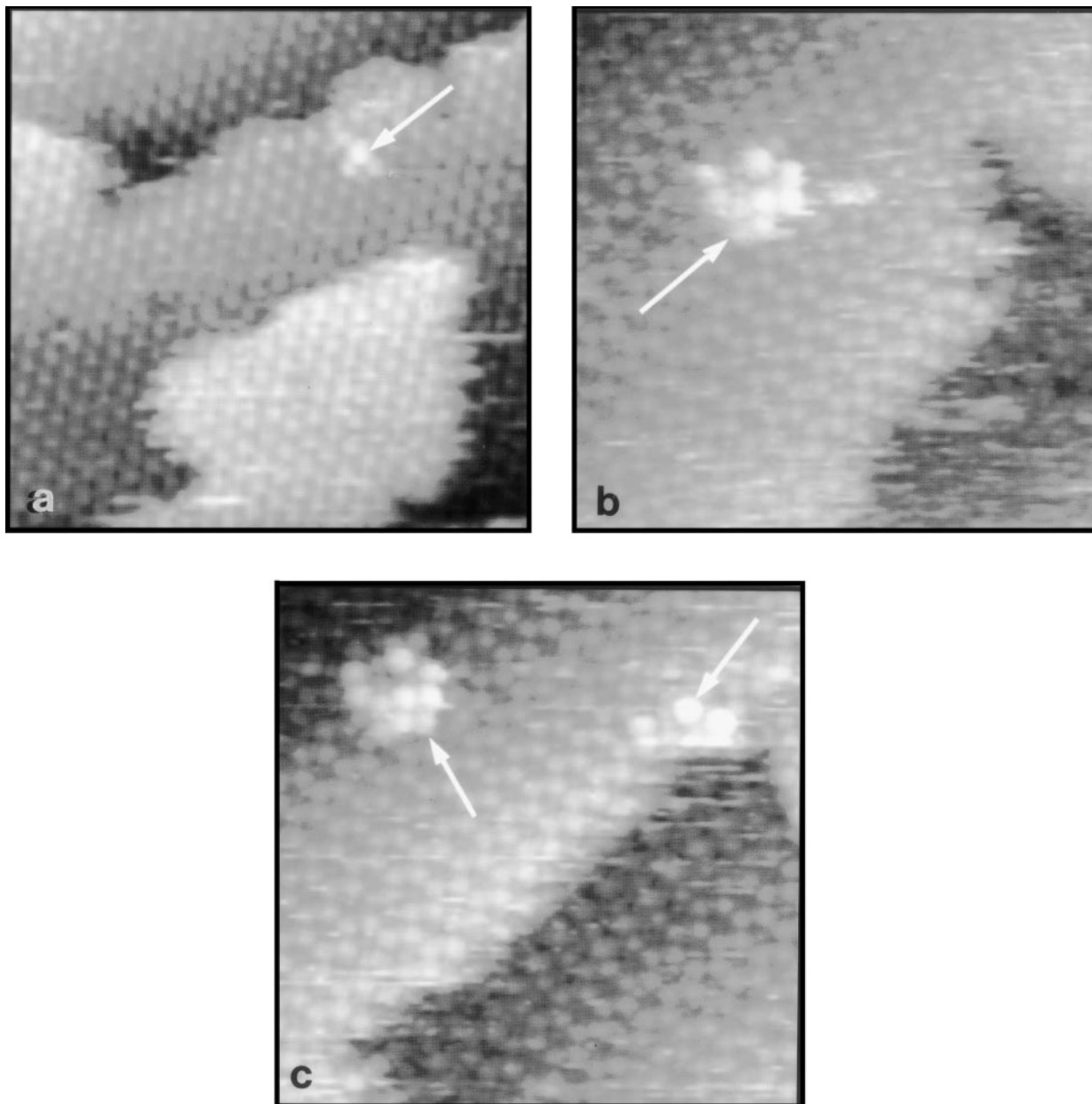
FIG. 3. (a) Individual virus particle (indicated with an arrow) adsorbed on the crystalline surface. (b) Cluster of nine virus particles (indicated with an arrow) adsorbed on the crystalline surface. In (c) a new virus particle (indicated with an arrow) incorporated into the cluster while several other virus particles (indicated with an arrow) assembled elsewhere on the surface. The scan sizes are (a) 1.3×1.3 µm² and (b and c) 800×800 nm².

however, rule out tip influence as a possible factor in promoting the transitions. AFM tip pressure could alter the chemical potential of lattice units, thereby causing particle release from the surface near equilibrium conditions. For other macromolecular crystals we have studied, more than a dozen (Malkin *et al.,* 1995a,b; Kuznetsov *et al.,* 1998), tip pressure produced visible scarring of the crystalline surface, but not an organized, cooperative restructuring as we observe here.

The pattern seen in Fig. 5a was initially observed on the (101) surface layer. Transformation then occurred to yield that seen in Fig. 5b, which subsequently restructured to yield the pattern seen in Fig. 5c. The relationship between the three motifs, and the mechanism of transformation from one to another, is shown in Fig. 6. Starting with the motif in Fig. 5a, and the corresponding Fig. 6a, removal of all particles of class A, which occupy specific locations in the crystallographic unit cells, yields the motif of
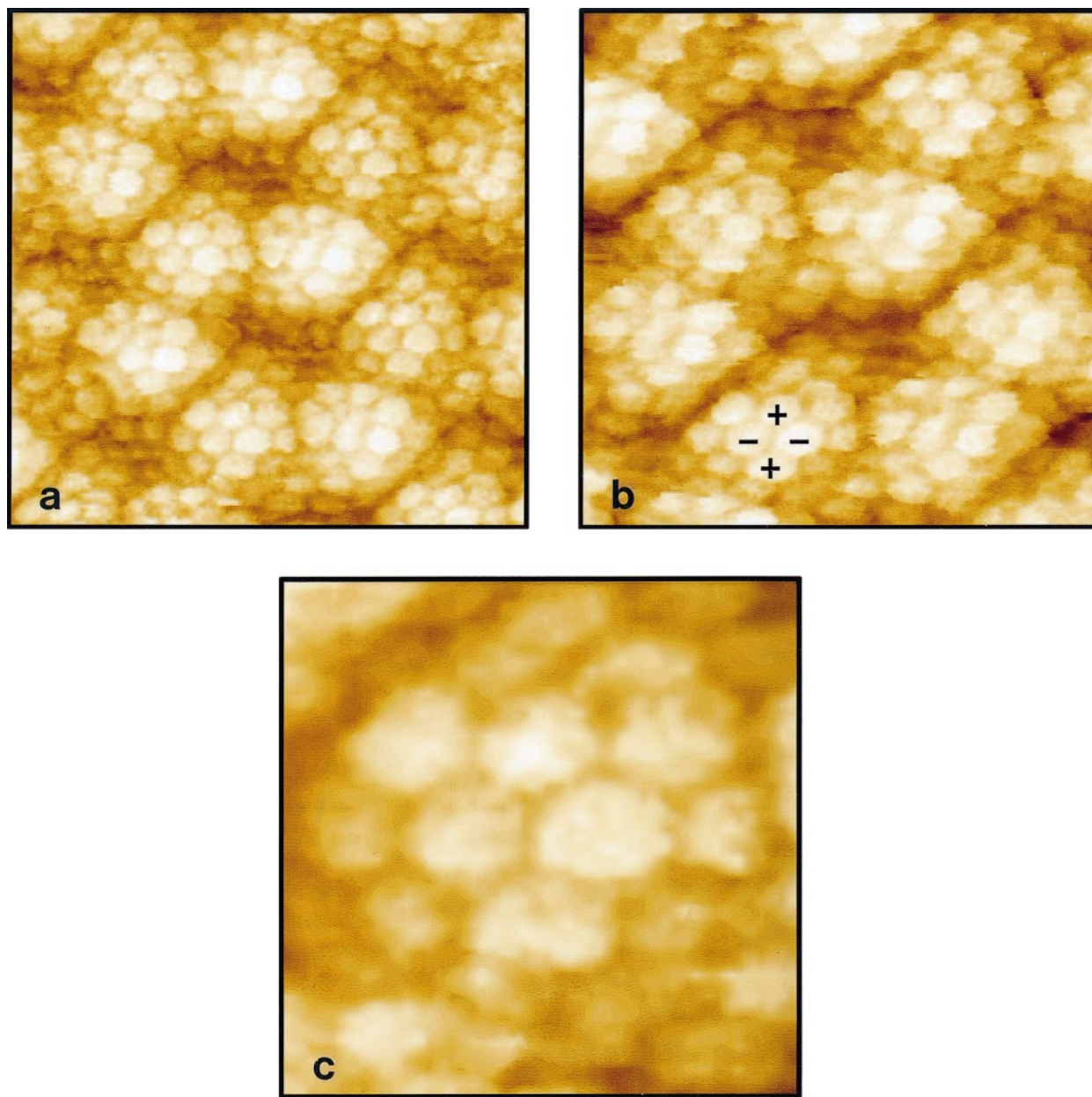
FIG. 4. *In situ* AFM images of TYMV particles immobilized in the crystalline lattice clearly display capsomers on the surface of the $T =$ 3 icosahedral virions. The capsomeres, from both X-ray diffraction (Canady *et al.,* 1996) and AFM, are roughly 60 Å across and protrude above the viral surface by about 45 Å. In (b) hexameric and pentameric capsomeres are indicated with minus and plus signs, respectively. The scan areas are (a) 140×140 nm^2, (b) 100×100 nm^2, and (c) 38×38 nm^2.

Fig. 5b and the corresponding Fig. 6b. Further removal of all particles of class B, as seen in Fig. 6b, produces the motif appearing in Fig. 5c, and in the corresponding Fig. 6c. The process of surface restructuring was not reversible and the structure presented in Fig. 5c was stable for prolonged periods over the course of experiments, which typically lasted for 6–8 h. Note that hexagons, which can be clearly seen in Fig. 5c, are formed by virions in positions $CA_1CA_1CA_1$ (Fig. 6c). Although there is

some height difference between virions in positions C and A_1 (Fig. 6c, bottom), it is not apparent in experimental images (Fig. 5c). The same is true for virions in positions A and C in Fig. 5a. This is because tapping mode AFM frequently does not produce entirely accurate height information compared with contact mode. Thus the transformations can be described as an organized emission from the lattice of distinct classes of virus particles occupying specific crystallographic lattice positions. In each
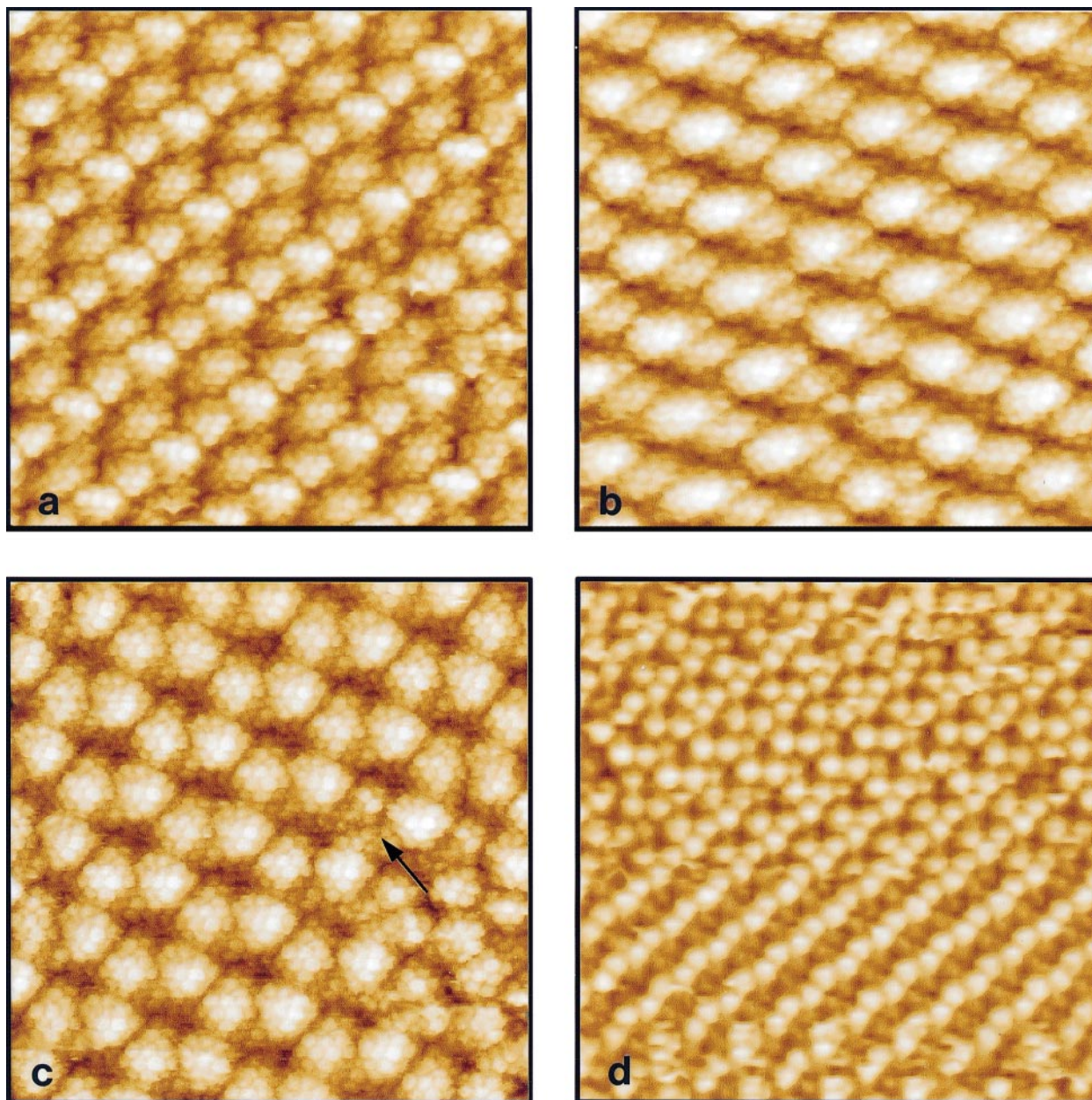
FIG. 5. (a–c) *In situ,* 300 × 300 nm² AFM images recording sequential transformations of the surface layer of the (101) face of TYMV crystals like those seen in Fig. 1b, when exposed to equilibrium conditions. In (d) is a 625 × 625 nm² AFM image of the surface layer that exhibits two of the three packing motifs of the TYMV particles. Vacancies (an example is indicated by an arrow in (c)) in the surface layer, where one or more individual particles are absent, were occasionally observed.

transition, the class of particles lost is that which protrudes highest above the surface and which maintains the least contact with the viruses forming the remainder of the crystal lattice. This is the class that is least firmly bound and has the highest chemical potential.

The salient feature of the surface transitions observed here by AFM is their organized, cooperative nature. Unlike normal dissolution, as seen particularly in etching experiments (Malkin *et al.,* 1996b;

Monaco and Rosenberger, 1993), the release of molecules from the crystals does not occur in a sequential manner, molecule after molecule, at step edges or at sensitive points of high chemical potential such as defects. Currently we are initiating additional studies on the influence of supersaturation and temperature on surface transitions in TYMV crystallization.

The results presented here show that, using tapping mode AFM, the capsomere structures of viruses

FIG. 6. Computer-simulated orthogonal images of the different surface structures of the (101) faces of the TYMV crystals based on the packing of the particle known from X-ray diffraction. Each class of particles A, B, and C corresponds to viruses occupying symmetry-related positions in the unit cell. Although these units are identical in the interior of the crystal, they are unique when in a surface layer. Sequential loss of particle class A from the motif in (a), corresponding to Fig. 5a, yields the motif in (b), corresponding to Fig. 5b. Subsequent loss of particle class B in (b) yields the motif in (c), which corresponds to that observed in Fig. 5c. In (c) particles in positions C and A_1 (from the next layer down) form the hexagonal rings of particles seen in Figs. 4a, 4b, and 5c.

can be reliably visualized and, to at least some extent, characterized. The correlation with X-ray diffraction at the same resolution is excellent. Because of the large size of the lattice unit, an entire virion, the crystals possess a number of unique properties when compared with conventional crystals and even with other macromolecular crystals. Furthermore, the large particle size allows studies of dynamic processes on the crystalline surface, opportunities not otherwise available. The surface restructuring presented here represents the first example of such a mass cooperative process that we have observed in any macromolecular crystals under study.

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