Atomic force microscopy of three-dimensional membrane protein crystals

Ca-ATPase of sarcoplasmic reticulum

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ABSTRACT We have observed three-dimensional crystals of the calcium pump from sarcoplasmic reticulum by atomic force microscopy (AFM). From AFM images of dried crystals, both on graphite and mica, we measured steps in the crystal thickness, corresponding to the unit cell spacing normal to the substrate. It is known from transmission electron microscopy that crystal periodicity in the plane of the substrate is destroyed by drying, and it was therefore not surprising that we were unable to observe this periodicity by AFM. Thus, we were motivated to use the AFM on hydrated crystals. In this case, crystal adsorption appeared to be a limiting factor, and our studies indicate that adsorption is controlled by the composition of the medium and by the physical-chemical properties of the substrate. We used scanning electron microscopy to determine the conditions yielding the highest adsorption of crystals, and, under these conditions, we have obtained AFM images of hydrated crystals with a resolution similar to that observed with dried samples (i.e., relatively poor). In the same preparations, we have observed lipid bilayers with a significantly better resolution, indicating that the poor quality of crystal images was not due to instrumental limitations. Rather, we attribute poor images to the intrinsic flexibility of these multilamellar crystals, which apparently allow movement of one layer relative to another in response to shear forces from the AFM tip. We therefore suggest some general guidelines for future studies of membrane proteins with AFM.

INTRODUCTION

Recent studies of biological samples with both scanning tunneling microscopy (STM)[1] and atomic force microscopy (AFM) have produced very exciting images of lipid mono- and bilayers (Marti et al., 1988; Meyer et al., 1991), DNA (Weisenhorn et al., 1990), soluble proteins (Edstrom et al., 1990), ion channels (Kolomytkin et al., 1991), amino acid crystals (Gould et al., 1988), and two-dimensional crystals of membrane proteins (Butt et al., 1990; Hoh et al., 1991). In some cases (Meyer et al., 1991) the resolution is very close to that obtained with other techniques (Vogel and Wöll, 1986), but in the case of membrane proteins (Butt et al., 1990; Hoh et al., 1991) the resolution is lower (Unwin and Ennis, 1984; Henderson et al., 1990), showing the present limitations of STM and AFM. These techniques image surfaces and can be used either for de novo structure determination or to supplement other structural studies of large macromolecules, like membrane proteins. For example, two-dimensional crystals of bacteriorhodopsin recently have been studied by AFM (Hoh et al., 1991) and the extramembranous loops that were imaged are not visible in three-dimensional reconstructions from electron micrographs (Henderson et al., 1990). However, to our knowledge, no study of three-dimensional protein crystals has yet been performed with AFM.

Ca-adenosine triphosphatase (Ca-ATPase) from sarcoplasmic reticulum (SR) is a transmembrane ion pump and has been studied widely, not only because it is involved in the regulation of calcium concentrations in muscle but also because it is a model for a large family of ATP-dependent ion pumps. Its reaction cycle is relatively well known (for a review see Inesi, 1985), and its primary sequence of amino acids has been determined (MacLennan et al., 1985), although its tertiary structure has only been studied at low resolution (Castellani et al., 1985; Taylor et al., 1986). Recently, three-dimensional crystals of SR Ca-ATPase have been obtained (Dux et al., 1987) and studied by electron microscopy (Taylor et al., 1988; Stokes and Green, 1990*a, b*) at 6 Å resolution. These crystals, which can be made thin and plate-like, comprise a stack of lipid/detergent bilayers with Ca-ATPase molecules protruding from either face (Stokes and Green, 1990*a*). These crystals currently are being used for three-dimensional reconstruction by electron microscopy, and AFM could complement this work in two ways: by measuring thickness of crystals and ultimately by mapping the surface structure of individual molecules. STM previously has been used to measure the thickness of two important transmission electron microscopic (TEM) specimens, though in this case they were two-dimensional crystals: purple membrane (Butt et al., 1990) and the bacterial HPI layer (Wang et al., 1990).

In the present study, we describe experiments aimed to observe these three-dimensional crystals of Ca-ATPase by AFM. We show that the expected morphology of air-dried crystals can be observed easily. Because crystals are poorly preserved by air drying—in fact, their periodicity is lost and molecular structure is probably de-

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[1] *Abbreviations used in this paper:* AFM, atomic force microscopy; ATPase, adenosine triphosphatase; $C_{12}E_8$, octaethylene glycol dodecyl-ether; HOPG, highly ordered pyrolytic graphite; MES, 2-(N-morpholino)ethanesulfonic acid; SR, sarcoplasmic reticulum; STM, scanning tunneling microscopy; TEM, transmission electron microscopy.

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stroyed—we were motivated to observe crystals under hydrated conditions. When hydrated, we show that crystal adsorption is controlled by the composition of the medium and by the physical-chemical properties of the substrate; this adsorption must be optimized for successful imaging by AFM, both to maximize the density of crystals on the substrate and to minimize crystal movement during imaging. In contrast to two-dimensional crystals of membrane protein or to lipid mono- and bilayers, these three-dimensional crystals are composed of a variable number of layers, and in this study we have been able to count these layers and to measure their thickness. We have also observed 40–50 Å thick flat sheets with holes, which we have interpreted as mixed bilayers of lipid and detergent.

MATERIALS AND METHODS

Ca-ATPase

SR vesicles were prepared and protein concentration was determined as previously described in Champeil et al. (1985).

Crystallization

Three-dimensional crystals of Ca-ATPase were obtained as previously described in Stokes and Green (1990*a*). Final concentrations were as follows: 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6), 100 mM KCl, 100 mM $CaCl_2$, 3 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 1 mM NaN_3, 2 mg/ml Ca-ATPase, and 1.8–2.2 mg/ml octaethylene glycol dodecylether ($C_{12}E_8$).

Electron microscopy

TEM was used to characterize negatively stained crystals and was performed as previously described in Stokes and Green (1990*a*).

Scanning electron microscopy was used to quantify the amount of crystals adsorbed to various substrates. A few microliters of crystals solution was put either on highly ordered pyrolytic graphite (HOPG) or on freshly cleaved mica (uncoated or coated; see below); after 1 min the substrate was washed and air dried and then sputter coated with gold.

Substrate treatment

Freshly cleaved mica was used both uncoated and coated with either carbon or polylysine (Fisher, 1982). Silicon wafers were cleaned by ultraviolet irradiation under oxygen flow.

AFM

The AFM was Nanoscope II (Digital Instruments, Santa Barbara, CA); the maximal scan range was $80 \times 80\ \mu m^2$. For all experiments, we used cantilevers with an elastic constant k 0.06 N/m. Typical forces were 10^{-8} N for experiments in air and 10^{-9} N for experiments under immersion. The microscope was always operated in the constant force mode.

Sample preparation

Dried samples were prepared by pipetting a few microliters of crystal solution onto the substrate, incubating briefly, rinsing in distilled water, removing most of this water with filter paper, and allowing the samples to dry at room temperature. The rinse in distilled water was essential to prevent uncontrolled changes in osmolarity and salt crystal formation during drying. Hydrated samples were prepared similarly, except that they were kept for 1 h in a petri dish placed on ice, washed with a variety of buffered solutions, placed in the microscope still covered with solution, and covered with a sealed liquid cell (Weisenhorn et al., 1989). Because AFM was performed at room temperature and crystals were grown at 4°C, we used negative staining and TEM to verify that crystal morphology and periodicity were retained after prolonged incubation (≥1 h) at room temperature in a variety of solutions, including distilled water.

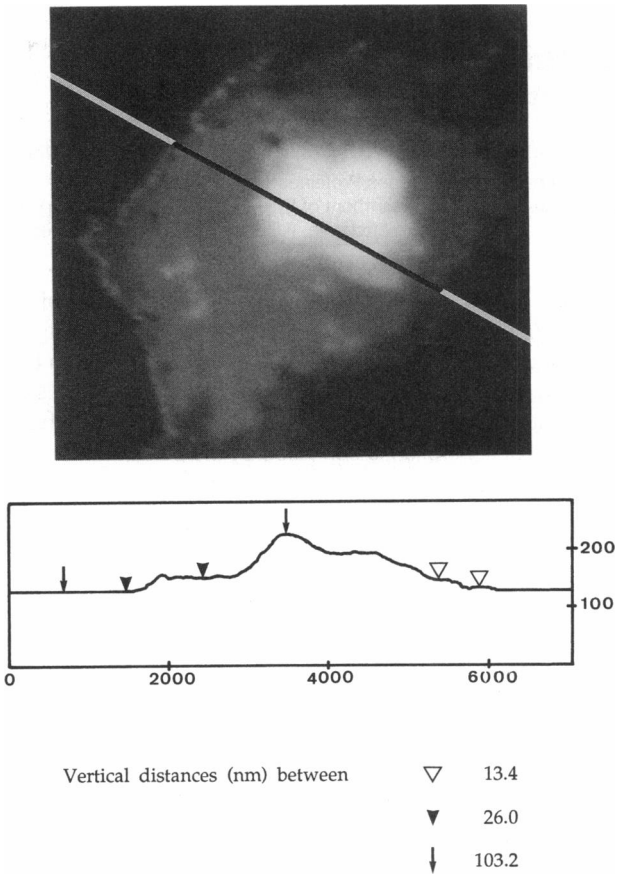


FIGURE 1 AFM image of a typical three-dimensional crystal that has been air dried on HOPG. The lower panel shows crystal height along the diagonal line drawn in the upper panel. Three pairs of arrows along the profile indicate locations for measuring differences in height, which are shown at the bottom. These height differences are consistent with a step size of ~13 nm, corresponding to the unit cell spacing for these air-dried crystals. Note also the ridge along the left side of the crystal, presumably caused by repeated scanning of the tip across the crystal. Crystallization medium was 20 mM MES, pH 6, 100 mM KCl, 3 mM $MgCl_2$, 10 mM $CaCl_2$, 20% glycerol, 2 mg/ml $C_{12}E_8$, and 2 mg/ml Ca-ATPase. After adsorption to HOPG, the sample was washed in distilled water to prevent formation of salt crystals on drying. The image is $6 \times 6\ \mu m^2$.

RESULTS

Dried crystals

We have used two different substrates for imaging dried Ca-ATPase crystals by AFM: HOPG, which has a hydrophobic uncharged surface, and mica, which has a hydrophilic negatively charged surface. Fig. 1 shows a group of crystals adsorbed to HOPG together with a height profile

(maximal height 115 nm). The steps in this profile correspond to integral numbers of unit cells, and the smallest step (13 ± 1 nm, $n = 20$) is comparable with the unit cell size measured by electron microscopy (16.4 nm) (Stokes and Green, 1990*a*), if we consider that for AFM, the crystals were dried in the absence of any preservative (e.g., negative stain). These observations were made on several crystal groups from two different samples (4 images in all), and they suggest that the large flat areas in Fig. 1 are two unit cells thick. In the case of HOPG, we have observed that after several scans, the edge of the crystal is pushed into a ridge, suggesting that adsorption of the crystals on HOPG is weak relative to the applied force. In the case of mica, we have observed an increase in the number of crystals adsorbed on the surface and have not seen this ridge at the edge of crystals, suggesting that adsorption was stronger. The density of crystals was still too low to use the high-resolution scan head, because it is limited to an area of $1 \times 1\ \mu m^2$. Nevertheless, using a low-resolution scan head of $80 \times 80\ \mu m^2$, we have been able to obtain images (similar to those in Fig. 3 *B*) showing an overall shape very similar to that observed with frozen-hydrated crystals in the TEM (see Fig. 3 *A*).

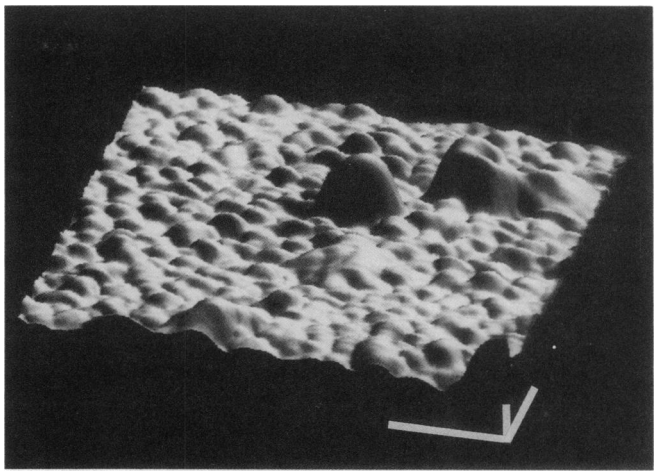


FIGURE 2 A surface representation of the mica after adsorption of crystals. The size of the image is $0.8 \times 0.8\ \mu m$ and an average "bump" is 60–80 nm in all three dimensions. Crystals have not adsorbed to this particular area, yet the surface is much more irregular than pure mica. The bumps have been identified as vesicles that are known to coexist with crystals and appear to have a very strong interaction with the mica surface. Sample adsorption was performed in the same way as Fig. 1, but, after rinsing with water, AFM imaging was done under immersion.

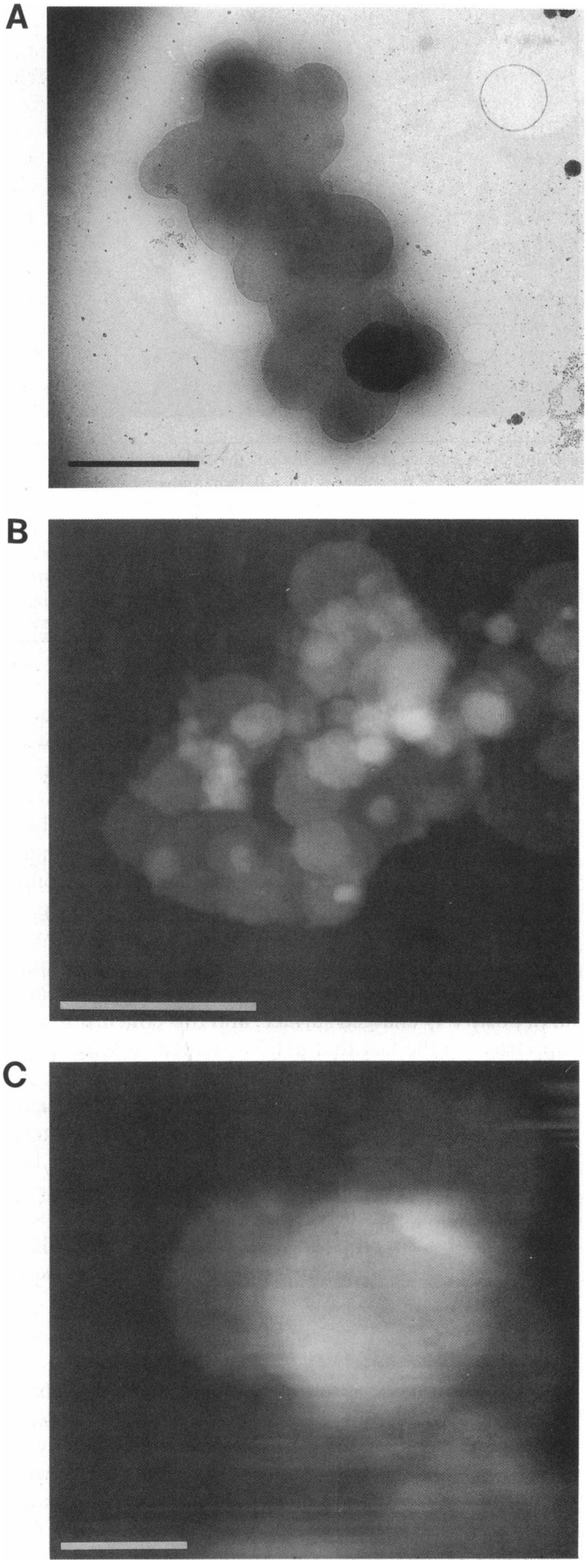
Preparation of hydrated crystals

Consistent with the observations of dry crystals, mica proved most useful for imaging hydrated samples by AFM. In our first attempt at AFM under immersion, the number of crystals adsorbed to mica was much smaller than that for dried samples. Furthermore, images were fuzzy and crystals were rapidly pushed out of the field of view by the scanning tip, suggesting that crystals did not adhere strongly to the substrate. We also observed an irregular background (Fig. 2), which did not resemble the surface of mica. Based on comparisons with similar images obtained from SR vesicles, we have identified this background material as vesicles (average diameter of 60–80 nm), which coexist with crystals in solution and which could be interfering with adsorption. This idea was supported by comparison of AFM images of a given sample, first hydrated and again after drying, showing a large increase in the number of crystals present after drying. Therefore, we next attempted to improve adsorption of hydrated crystals.

Ultimately, the best conditions involved removal of vesicles by three successive natural sedimentations of crystals (centrifugation at 1 *g*), followed by exchange of the crystallization buffer with the same buffer diluted fivefold. This procedure yielded virtually all of the crystals originally present in the solution, while removing almost all the vesicles as well as most solubilized lipid and protein (in detergent micelles). A concentrated suspension of crystals was then allowed to adsorb onto a freshly cleaved uncoated mica surface, and the mica was immersed in water to remove any nonadhering crystals; AFM imaging was done either in a dilute buffer (10 mM MES, pH 6) or in distilled water. We arrived at these conditions after studying the effects of vesicle removal, substrate charge, ionic strength, pH, and concentration of divalent cations by scanning electron microscopy. Freshly cleaved mica (negative surface charge) was a better substrate than either HOPG (hydrophobic and neutral surface), amorphous carbon (by evaporation onto mica), or silicon wafers (highly hydrophilic); coating of mica with polylysine, thus producing a positively charged surface, prevented crystal adsorption almost completely. This charge effect suggested that Ca-ATPase has a positively charged surface, and this conclusion was supported by the fact that pH 5 provided better adsorption than either pH 6 (crystallization pH) or pH 7. Furthermore, reducing ionic strength and removing divalent cations with EDTA increased adsorption, consistent with our hypothesis for an electrostatic interaction between Ca-ATPase and mica. It is noteworthy that crystals tended to aggregate after reducing both pH and salt concentration; therefore, images were recorded at pH 6 and low salt concentration as a compromise between crystal adsorption and aggregation.

AFM images of hydrated crystals

Using our optimized conditions for adsorbing crystals to mica, we observed both small and large groups of crystals with overall thicknesses up to 1 μm. The morphology of crystals in the smaller groups appeared to be better defined than previous attempts and were generally consistent with observations by TEM (e.g., Fig. 3, *A* and *C*). The background (i.e., the mica surface) appeared smoother for hydrated specimens (compare Figs. 2 and 3



C), presumably due to the removal of vesicles during the preparation. However, the concentration of adsorbed crystals was still too low to use the $1 \times 1\ \mu m^2$ scan head, and, with the $80 \times 80\ \mu m^2$ scan head, images of hydrated crystals (Fig. 3 *C*) were always fuzzier than those of dried crystals (Fig. 3 *B*). High magnification (i.e., small area) scans were uninterpretable, presumably due to excessive specimen movement. The observation of bilayers in these same preparations with much better resolution (see below) indicates that this fuzziness of crystal images was not due to instrumental limitations. Nevertheless, steps in the thickness of crystals, corresponding to the unit cell spacing along *C*, were clearly visible. On dry crystals, this spacing was measured at 13–14 nm, and the edges of groups usually contained individual crystals that were 2–4 unit cells thick; the central region was generally ~10 unit cells thick. On hydrated crystals, this thickness was measured as 16–17 nm ($n = 25$), and the number of unit cells was similar to dry crystals (based on 8 images from 3 different specimens).

In addition to crystals, the hydrated specimens also contained very thin flat perforated sheets (Fig. 4 *A*). The height of these sheets (Fig. 4 *B*) was 5 nm, which is consistent with the height of a lipid bilayer, and because the crystals are grown in sub-solubilizing concentrations of detergent, we reasoned that these perforated sheets could be mixed bilayers of lipid and detergent, with the edges of the bilayer being shielded by caps of detergent molecules. Recently, Singh and Keller (1991) also observed single bilayers on mica in a relatively similar situation. In their case, detergent was removed by dialysis from a solution containing solubilized lipid and mica. Although they observed no perforations, their bilayers had exposed edges that must have retained detergent caps. In our samples, we never saw vesicles or bilayers when HOPG was used. Because HOPG surface bears no charge, one may conclude that adsorption of vesicles and lipid bilayers on mica is mainly due to electrostatic interactions between charged headgroups of lipids (egg yolk phosphatidyl choline) and the surface of mica.

DISCUSSION

Three-dimensional crystals of Ca-ATPase, which are also being studied by electron microscopy (Stokes and

FIGURE 3 Comparison of electron microscope images (*A*) with AFM images of both air dried (*B*) and hydrated (*C*) specimens. For electron microscopy, crystals are rapidly frozen in their mother liquor and maintained in the frozen-hydrated state during imaging; a group of crystals is shown on a holey carbon support and the small black dots in the background are small particles of condensed ice. For AFM, vesicles were removed by allowing crystals to naturally sediment; crystals were then adsorbed to mica in 4 mM MES, pH 6, 20 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EDTA, 0.05% glycerol, 0.04 mg/ml $C_{12}E_8$, and 2 mg/ml protein and then washed with distilled water. AFM images of hydrated crystals were always "fuzzier" than those of dried crystals, presumably due to movement, even though forces were tenfold lower for imaging hydrated crystals. Scale bars correspond to 2 μm in *A* and *B* and to 0.5 μm in *C*.

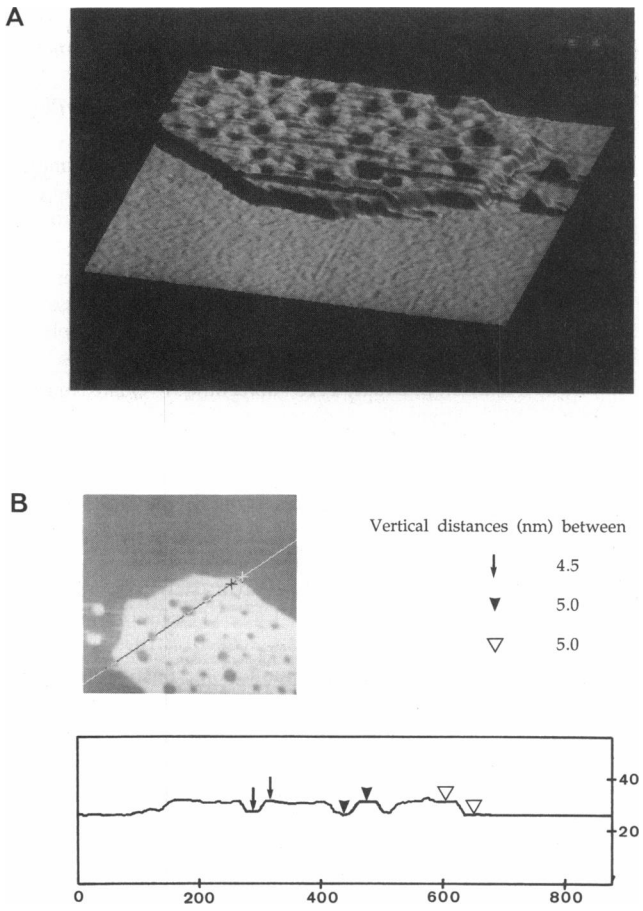


FIGURE 4 AFM images of a lipid/detergent bilayer under immersion. Despite removal of vesicles from crystal preparations, a few unilamellar structures are observed on mica after adsorption of crystals. *A* contains a surface representation showing a rather flat structure with occasional holes. The same particle is shown in *B*, together with a height profile along the diagonal line drawn. The thickness of this structure is 4.5–5 nm, corresponding to the thickness of a single bilayer. No Ca-ATPase molecules are seen, which would be 10-nm thick. The presence of detergent probably accounts for the holes and for the exposed edge of the bilayer by forming a cap around the otherwise exposed hydrophobic core of the bilayer. This particular bilayer is ~0.7 μm in diameter.

Green, 1990*a, b*), appear to be observable by AFM. Such observations require optimization of the physical-chemical conditions of crystal adsorption. Our experiments, performed with an electrically neutral substrate (HOPG) and with a negatively charged substrate (mica), show that adsorption involves complex interactions, such as van der Waals and/or electrostatic forces; comparison of images obtained with these two substrates show that electrostatic interactions are more effective than van der Waals interactions for adhesion of Ca-ATPase crystals.

Under the optimal conditions for adsorption, we obtained AFM images of two kinds of aggregates: the largest aggregates (maximum thickness of 1 μm) gave no structural information, whereas smaller aggregates (maximum thickness of 150 nm) allowed measurements of certain structural parameters. Even with small aggregates, edges of crystals always appear better defined than their centers, which are generally thicker. Dried samples, both on HOPG and on mica, gave images compatible with the overall shape seen by TEM. However, unlike TEM, AFM allows us to measure the thickness easily, thereby revealing the unit cell spacing normal to the plane of the substrate (13 ± 1 nm for dry crystals). Hydrated crystals also have been observed on mica by AFM, and in this case the unit cell spacing was 16–17 nm, consistent with x-ray diffraction of hydrated crystals (Stokes and Green, 1990*a*). The discrepancy is certainly an effect of drying, which is likely to cause collapse of extramembranous domains of Ca-ATPase that hold the unit cells apart. The ability to count the number of layers is relevant to current attempts at three-dimensional reconstruction by electron microscopy. This reconstruction requires knowledge of crystal thickness, and the current work represents a direct measurement of this thickness in images that are comparable with those obtained with frozen-hydrated samples, as well as to those from embedded/sectioned material (Misra et al., 1991).

In the case of both dried and hydrated crystals, we never obtained a resolution sufficient to observe individual Ca-ATPase molecules nor to measure periodicities in the plane of the substrate. This was expected of dried specimens based both on TEM, showing a loss of periodicity within the layers, and on AFM, showing evidence for collapse of extramembranous domains. We have used hydrated crystals to prevent this collapse, but the resulting images were always fuzzier than for dried samples. This fuzziness is likely related to horizontal displacement of crystalline layers induced by the application of force by the scanning tip, even with the lowest forces that could be used under immersion (10^{-9} N). This conclusion is consistent with our observations, on a given sample, that thicker crystals produce fuzzier images, even though lipid bilayers were observed with relatively good resolution (Fig. 4). Thus, we propose that in these three-dimensional Ca-ATPase crystals, the bonds between crystalline layers are relatively flexible. According to this hypothesis, the relatively high resolution (1 nm) images of bacteriorhodopsin (Butt et al., 1990) can be explained by the fact that these two-dimensional crystals consist of only one membranous layer, which is adsorbed directly onto mica. Furthermore, bacteriorhodopsin has almost no extramembranous domain, whereas in Ca-ATPase this domain represents ~70% of the molecule. Unfortunately, even on the thinnest Ca-ATPase crystals, which were two to four layers thick, there appears to be sufficient flexibility to prevent high-resolution imaging, presumably due to movements between these few layers. Imaging of monolayers would test this hypothesis, and Misra et al. (1991) claim to have grown unilamellar crystals. However, we have not attempted to reproduce these rather special crystallization conditions.

Higher resolution would require using unilamellar crystals or increasing the rigidity of three-dimensional

crystals, which could be performed by lowering the temperature. Alternatively, reconstituted bilayers of oriented Ca-ATPase could improve both the adsorption and the rigidity of specimens. Because Ca-ATPase is a very asymmetric molecule, one side of the reconstituted bilayer could be used for adsorption via lipid/substrate interactions, whereas the large cytoplasmic domains of Ca-ATPase could extend predominantly out into the solution and be imaged by the AFM. The feasibility of this idea is supported by our observations of strongly adsorbed vesicles (Fig. 2) and bilayers (Fig. 4), observations that recently have been confirmed by Singh and Keller (1991).

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