Atomic force microscopy of long DNA: Imaging in air and under water

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ABSTRACT We have obtained striking atomic force microscopy images of the intact λ bacteriophage genome and of several λ restriction fragments both in air and under water. The DNA is unstained and the images are stable under continuous scanning for up to 30 min. Measured contour lengths of fully imaged restriction fragments and intact λ DNA are accurate to within a few percent. The key to this development is the use of a process for binding unmodified double-stranded DNA to chemically treated mica surfaces. This procedure leads to strong DNA attachment and yields high-quality images that are stable under repeated scanning, even with the sample submerged in water. This allows normal hydration conditions to be maintained during scanning and in addition leads to a general improvement of image quality. Both the lateral resolution and the contrast increase by a factor of \approx 3 under water.

Scanning probe microscopy, which currently includes scanning tunneling microscopy (STM) and atomic force microscopy (AFM), is a structural tool ofgreat potential importance to structural molecular biology. AFM can image thick insulators, whereas STM is limited to small molecules (1, 2). Resolution is limited by the size and geometry of the scanning tip (3) , by the tendency of the tip to move the DNA $(1, 4)$, and by deformation of the sample (5) and scanning probe (6). Bustamante et al. (7) have overcome the problem of holding DNA in place for AFM scanning with an ionic treatment of the mica surface that allows DNA to be imaged in air under ambient conditions. Hansma et al. (8) have obtained improved results by imaging DNA submerged under propanol. The propanol eliminates forces due to the capillary condensation that occurs in ambient conditions, allowing less contact force to be used in imaging. Hansma et al. (8) and Bustamante et al. (7) also obtained improvements in resolution by using so-called "super-tips," which are individually fabricated and are much sharper than most of the AFM tips that are commercially available. Here, we report a procedure for chemical modification of mica so that it binds DNA strongly. Reliable images are obtained even in ambient conditions and with commercially available tips (which exert much more force on the sample; see below). We have scanned the same molecules in these conditions more than 60 times (more than an hour of continuous scanning) with no discernible change in the image. However, of most importance for biological purposes, the binding is strong enough that it is not necessary to use a nonsolvent (like propanol) to eliminate capillary forces. The DNA remains stable on the surface even when imaging is carried out in water.

Chemical methods for holding DNA in place have been proposed by us (9) and others (10) and implemented in ambient conditions for STM imaging (11) and AFM imaging (12-14). Our method for chemically modifying mica substrates uses covalently bound 3-aminopropyltriethoxysilane (APTES) followed by methylation and hydrolysis (12-14). In preliminary scans in air, this method has enabled us to obtain high-quality images of linear and circular DNA (12). In the work reported here, we show that the method can be extended both to unstained DNA molecules as large as the intact λ phage genome (48,502 bp) and to scanning DNA under water. DNA images obtained under water not only reflect normal hydration conditions but also show significantly improved lateral resolution over those obtained in air. It therefore appears possible to use AFM for direct conformational studies of nucleic acids and nucleoprotein complexes.

MATERIALS AND METHODS

The procedure for mica modification is described in refs. 12-14. Briefly, freshly cleaved strips of mica were left in the APTES atmosphere created by a small pool of APTES in the bottom of a 2-liter glass desiccator left at ambient temperature for 2 h. The methylation procedure was the same as described in ref. 14. The amino groups of APTES are bound covalently to a freshly cleaved mica surface, endowing it with properties similar to an anion exchanger.

Modified mica strips were immersed into DNA in Tris'HCl buffer (pH 7) (10 mM Tris.HCl/10-20 mM NaCl/5 mM EDTA) and incubated at room temperature for between ¹ and ² h. Concentration of DNA was varied between 0.01 and 0.1 μ g/ml. λ DNA and HindIII fragments of λ DNA were purchased from New England BioLabs and used without additional purification. After the adsorption stage had been completed, the samples were rinsed with deionized water (NanoPure water system, Dubuque, IA), blotted at the edge, and vacuum-dried.

Imaging was carried out on ^a NanoScope II STM/AFM from Digital Instruments (Santa Barbara, CA) using commercial AFM cantilevers (spring constant, 0.58 and 0.12 N/m) also from Digital Instruments. The tips were imaged using whisker crystals, as described elsewhere (15), yielding an estimate of the tip radius as 20-50 nm. The liquid cell used for AFM in this work was fabricated by Digital Instruments. The length of molecules is measured directly from the images using the public domain software NIH IMAGE 1.40.

RESULTS AND DISCUSSION

Before discussing our results, it is useful to review the interaction of an AFM tip with ^a substrate (1, 16). Atomic resolution is generally believed to arise from contact by a single atom asperity. Such contact forces decay even more

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Abbreviations: AFM, atomic force microscopy; STM, scanning tunneling microscopy; APTES, 3-aminopropyltriethoxysilane. *On leave from the Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia.

rapidly than tunnel current (17) so that the AFM should produce higher resolution than the STM. However, the nominal contact force measured by cantilever deflection F_N is the difference between the total (long range) attractive force F_A and the true repulsive force at the asperity atom F_R . We might model the tip as a sphere of radius R to which is attached an atomic asperity of diameter D. When such a tip is in contact with a flat surface, the total attractive force due to Van der Waals interactions between the sphere and the substrate is $-AR/6D^2$ where A is the Hammaker constant, typically 10^{-20} J for insulators interacting in water (18). If R = 20 nm and D = 2 Å, then $|F_A|$ = 1 nN. Thus, $|F_R|$ must be at least as big. Such a force, acting on an atomic asperity, creates a pressure greater than the yield strength of diamond (16) so that the tip will deform until it is stable. We have compared the resolution of the STM and AFM operated under electrolyte and imaging the same surface, and we find evidence of this effect (6). In ambient conditions, the force created by the meniscus of the water that condenses into the gap is usually orders of magnitude greater (perhaps hundreds of nanonewtons, see ref. 16). This force is also reduced when the tip radius is reduced. Thus, the use of "supertips" (7, 8) results in much smaller values for $|F_R|$ for a given $|F_N|$. However, the most important factor is elimination of capillary interactions by imaging under solution (water, in our case).

Images of fragments from the HindIII digest of λ DNA obtained in air are shown in Fig. 1A. Results are shown as raw data. All images are stable: After continuous scanning for 30 min at contact forces $(|F_N|)$ between 40 and 90 nN, no changes can be visually discerned. Although limited aggregation occurs in a few preparations, individual strands generally can be followed easily and measured strand lengths correspond well with the estimated lengths of the restriction fragments. The apparent height of the strands is $2-4$ Å (much less than the nominal 20 \AA) but the exaggerated apparent lateral width due to tip width broadening is evident in all strands as shown in Fig. 1A Inset (which shows one of the "strands" in Fig. 1A at higher magnification). In this case, the width of the image is nearly 800 A, considerably more than might be expected from our measurements of the tip radius. This suggests that a large deformation of the tip is occurring. Optical interference in the microscope generates the broad dark bands that cross the image vertically in Fig. 1A and contrast can be enhanced if these artifacts are removed by a high-pass filter. The effect of this is shown in the Inset in Fig. 1B, where the 23.1-kb HindIII restriction fragment is shown in its entirety. Its measured contour length is 7.5 \pm 0.3 μ m, which is close to the 8 μ m expected if the fragment maintains B-form base stacking (13) (A-form stacking would result in a contour length of $6.6 \mu m$).

The complete λ phage genome of 48,502 bp (14) is shown in Fig. 1B. This particular molecule appears unusually

FIG. 1. (A) A 10 μ m × 10 μ m AFM image of HindIII restriction fragments of λ phage DNA; raw data shows background fluctuations. (Bar = 2 um.) (Inset) Higher magnification image showing broadening (arrows point out strand width) and three-dimensional arrangement of strands. (Bar $= 0.25 \mu m$.) (B and C) AFM images (high-pass filtered) of whole λ DNA and various HindIII restriction fragments. (B Inset) Another AFM image of well-separated HindIII restriction fragments of λ phage DNA including the 23.1-kb fragment (marked with an arrow), high-pass filtered to remove instrumental background. All images shown in A-C are stable under continuous scanning at contact forces between 40 and 90 nN for at least 30 min. The apparent height of the DNA above the background is \approx 2.5 Å. The contour length of the molecule shown in C is measured from a series of higher-magnification images. An example is given in D, which is a high-magnification scan over the right side of the molecule shown in C.

FIG. 2. (A) A 10 μ m \times 10 μ m scan over λ phage DNA on APTES-treated mica under water. The fragment indicated by the arrow is an intact λ phage genome if it is looped as shown schematically in the Inset. (B) A 5 μ m \times 5 μ m scan at higher magnification of the molecule indicated by an arrow in A. The apparent height of the DNA above the background is ≈ 6 Å and the net contact force is 10 nN.

straight and stretched and its contour length is $15.7 \pm 0.7 \,\mu \text{m}$, which is very close to the \approx 16.4 μ m calculated assuming a B-form helical rise. Most of these large intact λ genome molecules are not as extended, however, and tend to be curved or even convoluted. A typical example is shown in Fig. 1C. In such cases, better measurements of contour length can be obtained from a set of higher resolution images of contiguous regions of the molecule. An example of one of these is the zoomed image shown in Fig. 1D. From a complete set of such images, we estimate the contour length of the molecule shown in its entirety in Fig. 1C to be $15 \pm 1.0 \mu m$, which is also in satisfactory agreement with that expected for intact λ phage DNA. The fact that images of these large DNA molecules are both stable and reproducible and give good values for contour length measurements makes AFM an attractive possible tool for physical mapping applications.

By using APTES-treated mica substrates, the DNA systems shown in Fig. ¹ can also be imaged by AFM under water. Fig. 2 shows scans of the preparation described above obtained when the sample is covered with water. Several fragments litter the surface. We have not found extended molecules such as the one shown in Fig. 1B, but we believe that the convoluted molecule indicated by an arrow in Fig. 2A is an intact λ genome. The contour length of this fragment (shown schematically in Fig. 2A Inset) is 17 ± 1 μ m, in agreement with the expected length. The most striking aspects of the images obtained under water are the increased resolution and enhanced contrast. Fig. 2B shows a scan taken at higher resolution over the molecule indicated by the arrow in Fig. 2A. Note that the molecule has remained stable when scanned again. The width of the image varies from 100 to 300 A, much less than the typical widths observed with these tips in air. The apparent height of the molecule is $5-7$ Å, an increase that roughly conserves the volume of the imaged molecule when compared with the data obtained in air.

These effects are illustrated most dramatically by imaging the same molecules with the same tip in air and under water. We show such a comparison in Fig. 3. These images were

FIG. 3. AFM images of λ DNA in air (A) and under water (B). The same cluster of molecules has been located under water (in B) and imaged with the same tip used to obtain images in air. The narrowing is evident when the regions indicated by arrows are compared. Conditions in air are as Fig. 1. Conditions in water are as in Fig. 2. The magnification of both images is the same and is indicated by the bar in A .

obtained using a liquid cell for the AFM. The image of Fig. 3A was first obtained in air, then the cell was filled with deionized water and the same region was scanned again (Fig. 2B). The latter is very similar to Fig. 3A, but, in many places, the width is substantially reduced, and the contrast substantially enhanced. We have averaged a number of cross-sections of these images, finding that the apparent height of the DNA is in $creased \approx 3$ -fold under water and the apparent width is reduced by a similar factor (the arrows on each image point to a region that narrows somewhat more than this). Studies of DNA under propanol made with supertips (8) showed even greater resolution and further enhancement of contrast. These effects are discussed more fully elsewhere (22).

We have shown in this work that appropriate chemical modification of mica substrates allows extremely stable highquality AFM images of DNA to be obtained, limited mainly by loss in lateral resolution occasioned by the large scanning tip diameter and the consequent interactions. It can be seen that using standard commercial AFM tips, the width of the image of double-stranded DNA is 10-40 nm. It is clear that much can be done to improve the resolution, for example, by use of sharper tips. Routine resolution in the 10-nm range would be higher than the best resolution presently achieved routinely by the cryo-electron microscopy of DNA and RNA (19). However, there are additional advantages of the AFM. (i) Our sample preparation is much easier, consisting only of substrate modification with no additional treatment (staining) of the sample. (ii) The sample can be prepared by deposition from a salt solution, which permits variations of the salt concentration and temperature of incubation in a broad range $(e.g., 0-60^{\circ}C)$. *(iii)* The stability of the binding permits studies under water (and, by extension, under buffer solution as well). These features are important for study of such fragile samples as complexes of DNA with proteins.

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