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Abstract
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DNA and RNA Polymerase/DNA Complex Imaged by Scanning Force Microscopy: Influence of Molecular-Scale Friction

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Summary: Reproducible images of DNA and the interaction of RNA polymerase with supercoiled DNA on mica have been obtained by scanning force microscopy (SFM) in air. This technique allowed visualization of the assembly of distinct nucleoprotein complexes at specific sites which initiate DNA replication. Transmission electron microscopic controls were performed on the carbon-coated replica of the same specimens previously scanned by scanning force microscope. Image contrast formation has been investigated and lateral friction forces have been qualitatively characterized with SFM on DNA-covered samples.

Introduction

The scanning force microscope (SFM) is designed to enable force measurements between a molecular-sized scanning tip and a surface, giving rise to a three-dimensional image related to the total density of electronic states up to Fermi level at the surface of sample (Binnig et al. 1986, Sarid 1991). In the setup, laser optical techniques (Meyer 1988) allow detection of the displacement of a few Å of a highly sensitive force sensor with small spring stiffness (Albrecht et al. 1990a). The deflection of the lever is a direct measure of the interaction forces between sample and tip. As the SFM can image soft surfaces in air or a liquid environment, with a lateral and vertical resolution down to 1 nm, forces as small as 10 nN in air or 0.1 nN in liquid can be measured (Burnham 1990, Weisenhorn et al. 1989). The scanning force microscope has proved to be an extremely valuable technique for imaging various biological materials adsorbed on flat surfaces (Gould et al. 1989, Häberle et al. 1992), often with molecular resolution (Butt et al. 1990, Hansma et al. 1992a). Several groups have attempted to image DNA by SFM (Hansma et al. 1991, Hansma et al. 1992b, Weisenhorn et al. 1991); however, one major difficulty lies in the preparation of the DNA sample (i.e., the deposition of DNA molecules on an atomically flat substrate in such a way that (A) the molecules do not aggregate and (B) they bind strongly enough to the surface). Therefore it is important to have a reliable and reproducible deposition and imaging method (Vesenka et al. 1992, Zenhausenn et al. 1992a,b).

Here we describe the procedure that we have used to visualize and analyze supercoiled DNA plasmid pUC9 adsorbed on mica and its interactions with Escherichia coli RNA polymerase by SFM in air. Our preparation method allows reproducible SFM imaging giving perfect consistency with our direct electron microscopic analysis by TEM. Although it was possible to corroborate measured molecular dimensions obtained by other biochemical and biophysical techniques, the structural information provided by our instrument was often limited by (A) tip geometric effects (Zenhausern et al. 1992c) and (B) by elastic deformation of the molecule due to high loading in air (Landman et al. 1990). Therefore, we use the SFM to investigate friction and adhesion forces at the molecular level on DNA and protein–DNA complexes. In particular, we show how frictional forces of a Si$_3$N$_4$ tip sliding over a molecular network of DNA adsorbed on mica surface can affect the apparent molecular dimensions and the image contrast.

Materials and Methods

The deposition method of double-stranded plasmids is a variation of a method described earlier (Zenhausern et al. 1992c).
A 6 µl drop of diluted DNA solution (pUC9, stock 2 mg/ml in 30 mM triethanolamine, pH 7.9, 10 mM MgCl₂, 0.1% glutaraldehyde) was adsorbed onto a small piece (1.5×1.5 cm²) of freshly cleaved mica (Marivac Ltd, Halifax) and air-dried. It was then washed in bidistilled water and dehydrated in ethanol prior to SFM observation. The binding of *E. coli* RNA polymerase molecules (holoenzyme preparation-Boehringer) to supercoiled pUC9 plasmid DNA (2673 base pairs) was performed as described by ten Heggeler-Bordier *et al.* (1992). The reaction mixture (0.13 mg/ml pUC9 in 30 mM TEA/HCl pH 7.9, 200 mM KCl, 10 mM MgCl₂, 2 mM DTT, 1° Pol I) was incubated for 20 minutes at 37°C and fixed with 0.2% glutaraldehyde for 15 minutes at 37°C. The complexes were then passed over a Sepharose 2B mini-column which was equilibrated with 30 mM TEA/HCl pH7.9, 10 mM MgCl₂ and 0.1% glutaraldehyde in order to separate the complexes from unbound RNA polymerases. The two most concentrated fractions were pooled and used in all adsorption experiments as described above.

SFM imaging was performed in air with a commercial system (SFM-BD2 from Park Scientific Instruments, CA) equipped with a piezo tube scanner with a scan width of up to 10 µm and installed on a vibration air-damped table. We operated with commercially available V-shaped cantilevers with an integrated pyramidal tip having a total length of 100 µm and 200 µm and spring constants of 0.37 and 0.032 N/m, respectively (Albrecht *et al.* 1999b). The deflection was monitored by reflecting a light beam of a laser diode (1 mW, 600–700 nm) from the end of the cantilever onto a two-segment photodetector. The linearity correction and calibration were obtained with a gold-coated optical diffraction grating supplied with our system. The atomic resolution on mica was easily obtained and we could measure the distorted hexagonal unit cell of muscovite mica with 0.28 and 0.35 nm between atoms on different sites (Emch *et al.* 1991). The size of the processed images was 256×256 or 512×512 pixels and no filtering was used.

For electron microscopy controls, a Pt-Pd film of about 2 nm was evaporated at 5°C on the specimens and coated with a 10 nm carbon film, then removed from the mica support by floating on bidistilled water and picked up with copper grids (400 mesh/inch, Balzers Union, FL-9496 Balzers, Liechtenstein). Specimens were investigated at 80 kV and a magnification of up to X 20,000, using a Philips CM-12 transmission electron microscope. The resulting electron micrographs were used to reveal equivalent structures also detected with our SFM.

**Results**

Reproducible structures along the double-stranded DNA adsorbed on mica surface have been resolved in details with our scanning force microscope. Figure 1A shows the 3-D conformation of pUC9 DNA molecules revealing kinks and loops due to the supercoiling of the plasmid. The presence of more relaxed DNA forms can be also observed (Fig. 1B). These conformations could be influenced either by ethanol dehydration process or by the interaction of DNA molecules with the mica surface. We determined the 840 nm averaged length of the molecule and a widened diameter of about 18 nm, due to geometric tip effect. TEM observation was used to test the feasibility of SFM imaging and therefore dimensions, shapes of plasmid DNA observed with SFM correlated well with the electron microscopic data obtained on the carbon-coated replica of the same specimen previously prepared and scanned with our instrument (Fig. 1C). These observations compared with SFM data allowed us to carry out a statistical analysis of the adsorption process of DNA on mica. The TEM replica in Figure 1D shows a homogeneous distribution of about 30±3 adsorbed molecules/µm², which is similar to the initial drop solution containing 29±5 molecules/µm². The supercoiled plasmids should bind to the mica surface by means of an electric double layer of adsorbed ions (Helmholz layers) allowing optimal conditions for SFM imaging in air.

Moreover, SFM was used to provide a direct visualization of the interaction of purified *E. coli* RNA polymerase molecules with the 2673 base pair long supercoiled DNA plasmid pUC9. There are two sites where the RNA polymerase is expected to bind the plasmid: (a) the promoter of the ampicillin resistance gene and (b) the ori site with promoter of two divergent transcripts directly involved in initiation of replication. With our instrument, we inquired about mapping of these site-specific bindings to DNA of RNA polymerase. As seen in Figure 2, most RNA polymerase are located at the terminal loops of the double-stranded DNA molecules. The same RNA polymerase distribution is shown by TEM in figure 2B. Under our experimental conditions, the DNA template has lost its supercoiled form in most cases as described in electron microscopic studies (Vollenweider *et al.* 1978); however, this conformational change did not affect the protein binding. This result indicates that DNA is still flexible during the alcoholic process and goes through successive forms after its adsorption on mica surface.

Figure 3A reveals a high distribution of RNA polymerase–DNA complexes adsorbed on mica substrate. The arrows indicate the site-specific binding of the protein to double-stranded DNA. The molecules of protein appear as spheres of about 15-20 nm in diameter, as measured on the cross-section shown in Figure 3C. The molecular heights vary from 3.5 to 6 nm. The resolution presently is limited by two factors: (1) geometry of the tip leading to broadened structures (Fig. 3D) and (2) tip–sample interactions. The loading force applied to the tip during a scan is detected from the force vs. distance curve (Fig. 3B). This hysteresis loop was generated by measuring the deflection of the cantilever as a function of the sample position. Minimal operating forces were typically in the range of 10⁻⁹ N in air in the repulsive mode.
Fig. 1  (A, B) Two typical SFM images of pUC9 plasmid DNA at various concentrations. In Fig 1A, an isolated double-stranded DNA molecule can be discerned and shows a twist due to the supercoiling of the plasmid. (C) Transmission electron micrograph of the carbon replica of the same specimen previously prepared for the scanning probe analysis demonstrating well the feasibility and accuracy of our SFM images. (D) Electron micrograph of a replica of plasmid DNA at an initial concentration of 29 ± 5 molecules/µm². A homogeneous distribution of DNA molecules is observed and about 30 ± 3 molecules/µm² are statistically detected. This permits maximization of the experimental conditions in relation to high binding of DNA molecules on mica surface. All electron micrographs were taken with a Philips CM-12 at magnification x55,000 comparable with SFM images.

Figures 4A, B illustrate how the rotation of the scan direction can influence image contrast in the case of DNA-covered samples. Figure 4A shows the variation of molecular height of DNA molecules as a sinusoidal function of the rotation angle. As seen, the contrast changes from positive to negative when the scan position is changed. In SFM images shown in Figure 4B, it is clearly demonstrated that the DNA molecules which appear with a positive contrast in the left picture after a scan rotation angle of −75° change to negative contrast in the right picture after a scan rotation of +15°. This effect can be interpreted, as illustrated with the model displayed in Figure 5, by considering frictional forces acting on the tip sliding over a DNA molecule adsorbed on mica. In the first case, if the sample is moving to the right, the tip is bent, thereby exerting a lateral friction force on the sample. When the sample is moving to the left (corresponding to a scan direction of 180°), the resulting forces reverse and image contrast changes. Notably the differential signal (A−B) of the detector is inverted yielding the change in contrast as seen in Fig. 4B. Further experiments are in progress in order to investigate this related friction effect quantitatively (Eng 1992).
FIG. 2 (A) Interaction of *E. coli* RNA polymerase to double-stranded pUC9 plasmid DNA was characterized by scanning force microscopy. Our study reveals two site-specific bindings to DNA of the protein. Supercoiled and relaxed molecules adsorbed on mica are observed. (B) Electron micrograph of the carbon replica of the same specimen previously scanned with our instrument. Images were taken at magnification ×55,000 on Kodak S0163 electron image film.

FIG. 3 (A) Top view SFM image of the RNA polymerase DNA complex. The arrows map the site-specific bindings to DNA of the protein. During this experiment, the contrast of the image switches to negative contrast, where the protein-DNA complex appears lower than the substrate due to tip-sample friction effect. (B) Force in arbitrary units (Y axis) versus distance between tip and sample in nanometers/division (X axis) taken in air. (C) Cross-section along the white line indicated in (A). The molecular dimensions of DNA (width: 50 nm, height: 2 nm) and proteins (dia. ~15-20 nm; height ~4-5 nm) can be estimated by considering various tip-sample interactions. (D) Model illustrating tip-geometry effect. The apparent lateral dimension of the molecule is given by the following expression: \[ L = 4.\sqrt{Rr} \] where \( R \) is the tip radius and \( r \) is the molecular radius. With the values \( R = 20 \text{ nm} \) (from SEM measurements) and \( r = 1 \text{ nm} \), the width is 17.8 nm. This result is comparable with experimental data obtained in Fig. 1a and b.
Discussion and Conclusion

The method described for preparing RNA polymerase-DNA complexes adsorbed on mica was easy to use, fast, and reproducible for SFM imaging. It was possible to detail the site-specific binding to double-stranded DNA of the protein, which could be implicated in the initiation of the DNA replication (Bordier and Dubochet 1974). We identified approximately the lateral and vertical dimensions of both the protein and the DNA plasmid. The RNA polymerases appeared as spheres of 15–20 nm in diameter and 3–6 nm high, while plasmids were about 2 nm high and 18 nm wide. Although other studies reflect more precise structural information (Tichelaar et al. 1983), the broadened features with reduced heights, which we observed with our instrument, can be explained by molecular distortions due to the various interactions forces between tip and soft biological materials. When operating in repulsive mode at ambient conditions, both the tip and the sample may be damaged. For example, elastic deformation of the surface of the sample under the applied force and the presence of water and an organic contamination layer on top of tip and specimen surface may modify the tip-sample interactions (Pethica and Sutton 1988). Most often, this will cause an enhancement of the tip radius during a scan cycle resulting in a major decrease of resolution. Therefore, the apparent lateral dimensions of the biological features are “tip geometry” dependent (Keller 1992).

Some authors (Hansma et al. 1992b, Vesenka et al. 1992) have shown that the relative humidity may influence on image formation by enhancing the meniscus force (capillary forces) which could distort DNA molecules. In our SFM experiments, we showed that an inversion of the image contrast can occur when the scan direction is rotated. We suppose that this effect is caused by lateral friction forces formed by dragging the tip along the surface leading to a distortion of the DNA plasmids. Therefore, our aim was to evaluate the influence of lateral friction interactions involved in SFM imaging. Such forces can be also detected in the repulsive region of the force versus distance curve where large hysteresis effects are observed (Fig. 3B). In this way, the heights of the features can vary and the image contrast becomes strong dependent on frictional forces. Thus, changing the scan direction by a large amount causes an abrupt change in contrast.

In conclusion, our SFM analysis provided a visualization of molecular-scale friction effects which appear implicated in image contrast formation. Thus, large frictional forces should be considered in the interpretation of SFM images. The observation of three-dimensional structures like protein-DNA complexes and the mapping of the site-specific bindings to DNA with SFM bring new exciting ways to study the DNA conformation in relation to its sequence.

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