MEASUREMENTS ON SINGLE DNA MOLECULES

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0. Abstract
All our understanding of how DNA works is based on evidence obtained with bulk methods. Moreover, information on the detailed conformation of DNA and interactions between DNA and proteins at a high resolution, have been obtained almost entirely under highly non-physiological conditions (vacuum, low temperature etc.). Recently several methods have become available that make it possible to study individual bio-molecules under physiological conditions. We will describe three of these methods and there application to DNA research: flow analysis of individual molecules, measuring mechanical forces of bio-molecules using optical tweezers and using an AFM to study the dynamics of DNA and DNA protein interactions.

Keywords: optical tweezers, DNA overstretching, denaturation, AFM, photolyase

1. Introduction

Probably DNA is the most important molecule for life on Earth. Although we precisely know how the genetic information is stored in DNA, little is known on the functional aspects of DNA. In a cell, the DNA is condensed by a factor of 10,000, in a very well organized way so that the important genes are still available for transcription. It is of great importance to understand how this is achieved. Numerous proteins are involved in this. Besides such structural aspects, various active processes take place on the DNA molecules, such as replication, gene transcription, damage recognition and damage repair. For all these processes, highly specialized proteins are active, and although our knowledge of these processes is rapidly improving, we are still at the beginning of our understanding. For an excellent introduction on this subject we refer to the book of Calladine and Drew [1]. DNA and DNA-protein interactions have been studied with a variety of methods, including various forms of spectroscopy, gel electrophoresis, and very importantly, X-ray diffraction. Although these techniques have yielded very important information, all these techniques are bulk techniques and thus represent the average properties of an ensemble of molecules. Recently, new technologies have been developed that enable us to study individual molecules under physiological conditions. In this article we will describe three examples that are currently studied in our laboratory.

2. Results and Discussion

2.1 Analysis of single molecules in flow

This technique is based upon a flow cytometer [2], that is optimized for the measurements of extremely weak fluorescent signals (see figure 1). The individual particles that will be measured are dispersed in liquid and are forced to flow in the middle of a small flow cell. This is achived using the principle of hydrodynamic focussing [3]. In the flow cell the particles are illuminated by a focused laser beam and the fluorescent light of the particles is

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measured. Individual DNA molecules can be measured in this way provided that they are labeled fluorescently with strong dyes such as TOTO-1 [4]. This intercalating dye stains DNA base-independently in a stoichiometric manner, so that the fluorescent signal is proportional to the length of the molecule. In figure 2 a typical example of a measurement is shown. In this case the sample consists of a particular DNA molecules, the so called λ DNA isolated from a *E. coli* bacteriophage, that is cut in a specific fragments using the enzyme Hind-III. This enzyme cuts DNA at places where the sequence is double AA. In this way fragments characteristic for the DNA are obtained. Thus the size distribution yields a 'fingerprint' of the original DNA molecule.

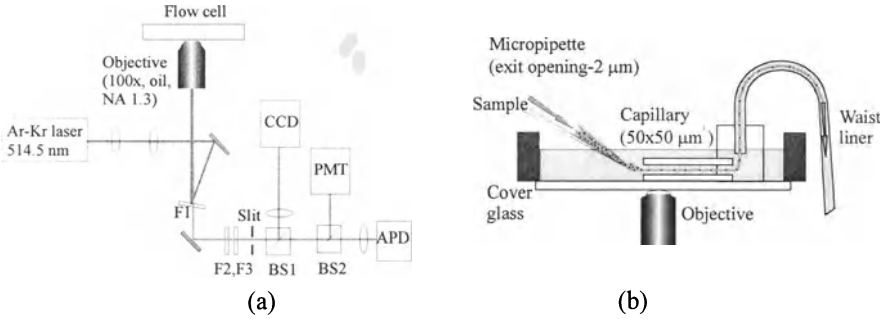

Figure 1. *Experimental set-up (a) to measure individual DNA molecules in a hydrodynamic focussing flow cell (b) CCD: image intensified CCD camera; PMT: photomultiplier; APD: avalanche photodetector.*

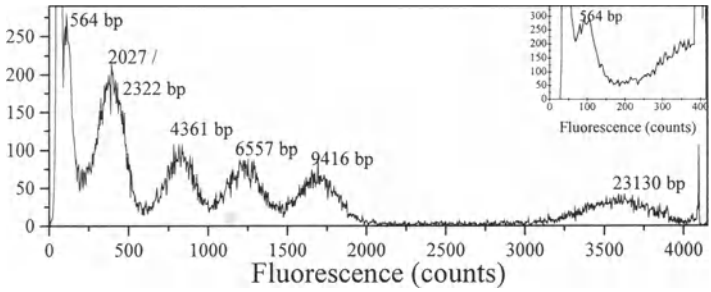

Figure 2. *Histogram of the fragments of λ DNA molecules after it was cut into pieces by the enzyme Hind-III.*

This method can be used for DNA sizing of DNA fragments ranging from 1.5 to 167 kbp (thousand base pairs). The lower limit is determined by the background signal from the water and can be improved by pre-bleaching the water, discriminating Raman scattering from fluorescence using time gated fluorescence measurements [5] and by decreasing the measuring volume. If the measurements can be automated and the sample handling can be improved, the method could be a alternative for gel electrophoresis with greatly improved analysis time, since the analysis can be performed in a few minutes. Extending this analysis to more parameters, e.g. measuring a specific DNA probe equipped with a fluorescent label that

can be spectrally separated from the TOTO, could open a new area of DNA analysis including rare event detection of specific genes.

2.2 Measuring mechanical properties of individual DNA molecules

In 1986 Ashkin et al [6] showed that small micron sized beads could be trapped at a stable position using a tightly focused laser beam. The technique, referred to as optical trapping or optical tweezers, has been successfully applied on a variety of biological samples [7,8]. Recently, Smith and coworkers have used this technique to measure the mechanical stretching properties of single DNA molecules [9]. Here we describe similar experiments done in our laboratory (figure 3).

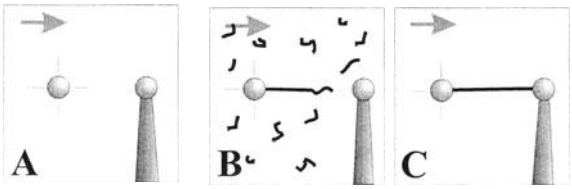

Figure 3. *Individual DNA molecules are connected to two different polystyrene spheres of 2 micron diameter using an optical trap and a glass micropipette with a 1 micron tip diameter. A: One streptavidin coated bead is trapped in the optical trap (indicated by 4 lines) and a second is hold by the glass micropipette. B: Buffer containing biotinylated DNA molecules is flown in the flow cell and one single molecule hits the trapped bead and attaches to it with one end. This can be detected by an increase of the force exerted on the trapped by due to the increased drag force. C: The second bead (on the pipette) is moved toward the open end of the DNA molecule to finish the system.*

The λ-DNA molecules which consist of 47,502 base pair are modified such that both ends of the molecules are biotinylated. Polystyrene beads with a diameter of 2 μm are flown into a small flow cell where a single bead is trapped using the optical trap. This bead is then transferred to the glass micropipette. A second bead is captured in the trap and the other beads are flown out of the flow cell. The buffer is now changed to a buffer containing the biotinylated DNA. A single DNA molecule in flow will hit the trapped bead and attach to it with one end. The next step is to 'fish' around with the bead on the glass micropipette, in order to get the open end attached to this bead. Now the system is ready to measure a force extension curve of an individual DNA molecule by moving the micropipette with respect to the laser trap position. The extension of the DNA molecule is determined as the distance between the beads using microscopic imaging. The force exerted on the DNA molecule can be obtained by measuring the small displacement of the trapped bead out of the laser trap. After calibration this directly yields the absolute force exerted on the molecule. The resulting curve (figure 4) shows an unexpected behavior of the elasticity properties of the DNA molecule. The behavior up to 18 μm is rather straight forward. Up to 16 micron the DNA is not completely stretched and the behavior can be described taking the entropy of the DNA molecule into account [10]: a fully stretched DNA molecule is subject to thermal fluctuations, which cause it to decrease it length. Therefore, a force is necessary to extent the DNA just to its full length of 16.4 μm. From this value up to 18 ì m, the DNA behaves as an elastic spring.

At a length of 18 micron, and a force of 65 pN, the molecule suddenly can be extended further to about 170 % of its normal contour length with only very minor effort. Stretching

beyond this point the DNA eventually again behaves like a spring. The entire curve is not completely reversible. If we relax the same molecule again the force at certain lengths will be lower than when we stretched the molecule. This indicates that the conformation change of the molecule is not completely reversible. Different models for the structural conformation change has been proposed, but the exact nature of the overstretched conformation is not known yet [9,11].

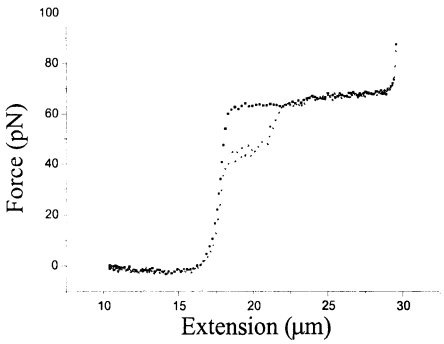

Figure 4. *Force extension curve of a double-stranded DNA molecule in buffer with 0.1 M NaCl. Black curve: Stretching. Gray curve: relaxing*

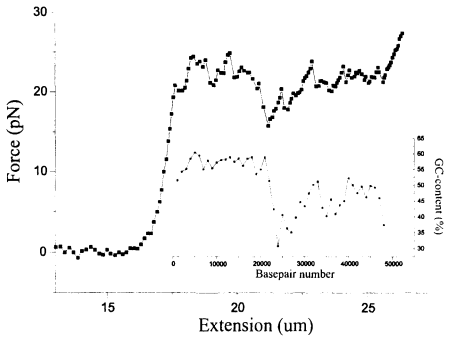

Figure 5. *Force extension curve of a double-stranded DNA molecule at low salt concentration. Insert: GC-content of λ-DNA as a function of position.*

If DNA molecules are brought into a buffer with a lower salt concentration, it is known that the DNA melting temperature decreases. The helix structure of the molecule is not stabilized anymore. We now build the system as described above and changed the buffer to distilled water in which no ions are present, and repeated the stretching of the molecule. The force-extension curve obtained is shown in figure 5. Up to a force of 20 pN the curve seems to be the same as in 100 mM Na Cl TE buffer, but after this the curve shows a very irregular behavior. This is interpreted as the actual dissociation ('melting') of the two strand of the DNA molecule. The 'melting' part of the curve seems very noisy, and arbitrary, but when we compare this with the GC-content of the DNA molecule, we observe a striking correlation (figure 5, insert).

2.3 Atomic Force Microscopy of DNA protein interaction

A very powerful method to study single molecules is the atomic force microscope, AFM. For biological applications, the ability to obtain images with a resolution about 20 nm seems to be extremely useful. However, under normal operation conditions, the tip will just move the biological molecules laterally across the surface. It was only after it was demonstrated that the AFM can also work with the much more gentle tapping mode in liquid that delicate biological samples could be studied [11]. We have shown that by careful optimizing the conditions of the sample and the instrument, the dynamic behavior of DNA molecules loosely bound to a mica surface, and the interaction with a individual protein molecules can be visualized with a series of AFM images. This is illustrated by the series of images below.

As AFM is a surface technique, the reactions have to proceed on a surface. In fact a paradoxical problem has to be overcome. To be able to image DNA reproducibly and with

high resolution it is necessary that the DNA is attached to the surface. Otherwise the DNA molecules would be swept away when the tip taps on the molecules and the image would be severely distorted. However, to be available for interaction with other molecules, DNA must be free from the surface to avoid steric hindrance that might affect the reactions. Thus DNA should be immobilized just firmly enough to allow both imaging with AFM and interaction with proteins.

In this study formation of non-specific complexes of dsDNA with photolyase is presented. Photolyase is a well-studied enzyme responsible for the removal of thymine dimers in DNA. The crystal structure was resolved [12,13] and structural features have been related to results from photochemistry. Although the reaction has been extensively described biochemically, AFM can contribute to a better understanding of the dynamic aspects of the reaction such as the process of locating the damaged site.

Freshly cleaved mica discs (Ted Pella, Ca) were used as a substrate for immobilized DNA. Undamaged 500 bp dsDNA (Gensura, Ca), obtained by digesting plasmid DNA with EcoRI, was diluted to a final concentration of 2 ng/µl in a buffer containing 4 mM Hepes, 1 mM NaCl and 1 mM MgCl2, pH 6.5. The buffers were made in MilliQ filtered deionized water. Photolyase, obtained from Anacystis Nidulans, [13,14] was added to a final concentration of 0.12 ng/µl in the reaction buffer.

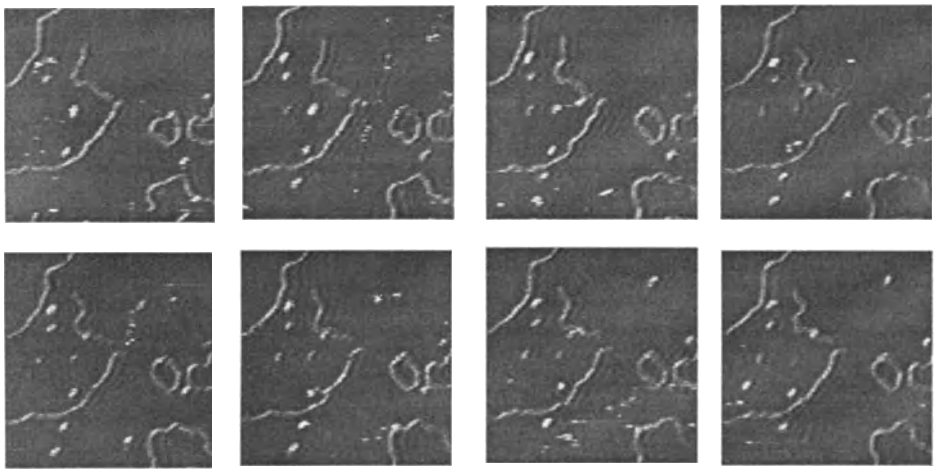

Figure 6. *Eight frames from a 30 minutes sequence of topography images obtained by tapping mode AFM at 1 frame per minute. The scan area was 0.5 x 0.5 µm^2, z-range 4 nm.*

After 10 minutes at room temperature 5 µl of the reaction mixture was deposited on mica. Directly after deposition the AFM was mounted over the sample and after a minute the liquid cell of the AFM was thoroughly rinsed with Hepes buffer, without DNA and photolyase. During sample preparation, mounting of the AFM and measurements, bio-molecules remain in buffer and are never dehydrated in order to keep them functional.

Figure ## shows 8 frames from a typical time series measurement of a photolyase DNA mixture. In this measurement the surface was scanned during 30 minutes at 1 frame per minute. During the 30 minutes of scanning no damage to the sample was visible, while the RMS roughness in the height of the atomically flat mica amounted less than 0.2 nm.

Acknowledgments
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