BIOPHYSIQUE À L'ÉCHELLE DE LA MOLÉCULE UNIQUE
SINGLE MOLECULE BIOPHYSICS

Mechanical opening of DNA by micro manipulation and force measurements

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Abstract
In this paper we summarize part of our work on the mechanical unzipping of DNA. We have prepared molecular constructions which allow us to attach the two complementary strands of one end of a single DNA molecule of the bacteriophage λ separately to a glass microscope slide and a microscopic bead. In a first series of experiments, a soft microneedle acting as a force sensor is attached to the bead and its deflection is measured with an optical microscope. In a second series, we use an optical trapping interferometer to capture the bead and to measure its displacement to nm resolution. The sample is slowly displaced with respect to the force measurement device, leading to a progressive opening of the double helix. The force measured during this mechanical opening shows a characteristic variation which is related to the base pair sequence of the DNA molecule. ***To cite this article: U. Bockelmann et al., C. R. Physique 3 (2002) 585–594.*** © 2002 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

micromanipulation / optical tweezers / DNA unzipping

Ouverture mécanique de la molécule d'ADN par micro-manipulation et mesure de force

Résumé
Dans cet article nous résumons une partie de notre travail sur l'ouverture mécanique de l'ADN. Nous avons préparé des constructions moléculaires qui nous permettent d'attacher les deux brins complémentaires d'une extrémité d'une molécule unique d'ADN du bactériophage λ, séparément à une lamelle de microscope et à une bille microscopique. Dans une première série d'expériences, un levier souple de verre, agissant comme capteur de force, est attaché à la bille et sa deflexion est mesurée avec un microscope optique. Dans une seconde série, nous utilisons un piège optique interférométrique pour capturer la bille et mesurer son déplacement avec une résolution nanométrique. L'échantillon est déplacé lentement par rapport au système de mesure de force, induisant une ouverture progressive de la double hélice. La force, mesurée pendant cette ouverture mécanique, montre une variation caractéristique qui est reliée à la séquence des paires de base de la molécule d'ADN. ***Pour citer cet article : U. Bockelmann et al., C. R. Physique 3 (2002) 585–594.*** © 2002 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

micromanipulation / pinces optiques / ouverture de l'ADN

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DOSSIER

1. Introduction

Both in physics and biology, molecular systems are usually studied by measuring ensembles of many individual molecules. This mostly has practical reasons, e.g. in optical spectroscopy the overall signal is proportional to the number of molecules interacting with the incident light. There is, on the other hand, information which is accessible only by non-averaging techniques, performed on very few or even a single molecule. These techniques may reveal details which otherwise would be masked by statistical fluctuations. Regarding the measurement of interaction forces, the precise control of the number of bonds contributing to the measured force is obviously necessary; otherwise, no quantitative statement is possible on the force associated to an individual bond. During the last years, an increasing number of single molecule force measurements have been conducted on biological molecules. In particular, the field of force measurements on single DNA molecules is presently very active [1–14]. Our experiments [6–8,15,16], where we pull open isolated double stranded DNA molecules by micromanipulation and measure the associated forces, combine both above mentioned advantages:

 (i) measuring the force while pulling on a single molecule we can conclude that the double helix mechanically opens at a force of the order of 10 pN,
(ii) the absence of statistical averaging over different molecules allows us to resolve a dinstinct dependence of the measured force on the genomic sequence of the DNA.

A number of theoretical papers have been published which directly relate to our experimental configuration [17–21]. Two other groups have reported on AFM measurements of average forces to separate the two strands of the DNA double helix [2,12], however, without resolving a base sequence induced variation in their force signals.

2. Experimental techniques

2.1. The molecular constructions

In order to address separately the two strands of a single DNA double helix, we have to prepare a special molecular construction. For the molecule to be opened a bacteriophage λ DNA (double strand contour length of 16.2 μm, 48 502 base pairs) is used. During the inital stage of our work [6–8], we functionalized one of the strands with a single biotin terminal group and prolongated the other one by a long linker arm, the latter being terminated by a single digoxigenin group. The linker arm was a full length double stranded λ DNA molecule.

The molecular construction (Fig. 1) used in our more recent studies [15] contains two linker arms rather than one in order to reduce as far as possible non-specific interactions between the molecule to be opened and the solid surfaces (microcope slide and silica bead). Also, multiple attachment points are introduced at each extremity of the construction to increase the stability of the molecule-surface linkages. Two different types of double stranded DNA, each about 2000 base pairs (bp) in length, are prepared by PCR, one with digoxygenin modified bases, the other one with biotin modified bases. From this, cohesive DNA fragments of about 1000 bp are obtained by digestion with an appropriate restriction endonuclease. Circular pTYB1
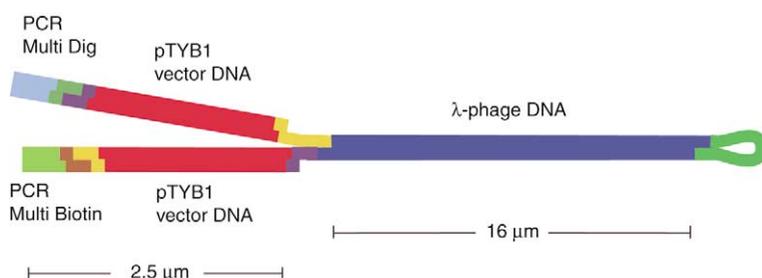
Figure 1. A molecular construction containing two linker arms and multiple attachment points of two different chemical type.

vector DNA (7280 bp) is linearized by double digestion. One extremity is ligated to the PCR product, the other extremity is ligated to a short synthetic DNA fragment formed by hybridisation of two partial complementary oligonucleotides. This way we create two different types of linker arm molecules each having either a digoxygenin or a biotin modified PCR fragment at one extremity and a short nonpalindromic single stranded overhang at the other extremity. The ds-DNA to be opened is a linearized λ-DNA with one extremity capped by a short hairpin oligonucleotide. In a final ligation step, the three parts, the two different linker arms and the DNA to be opened, are covalently assembled. Purification on spin columns or by phenol/chloroform/ethanol precipitation are performed where appropriate.

A small amount (of the order of 10^{-17} mol or 10^7 molecules) of the molecular construction is deposited on a glass microscope slide in a region limited by a small plastic ring. The slide has been coated with antibodies against digoxigenin (antidig) and specifically anchors the molecules through dig-antidig bonding. Afterwards, streptavidin coated silica beads are introduced that react with the biotin functionalized part of the molecular construction. The sample is covered by a circular glass slide (this step is omitted in the preparation of the micro-needle measurements) and placed on an inverted microscope. We apply an air flow at an oblique angle to the cover slide which induces a rotation and a liquid flow. A correctly attached bead thus moves around a single anchoring point with a tethering length of about 5 μm corresponding to the total length of the two linker arms (about 15 μm for the construction with a single λ-DNA linker). This allows us to select the beads that are correctly attached for the subsequent force measurements.

2.2. The force measurement devices

We use two different types of force measurement devices: biotin functionalized glass micro-needles that stick to the streptavidin beads or a trapping interferometer which traps the beads through a force induced by the spatial gradient of a tightly focussed optical wave-field.

Fine glass micro-needles (tip diameter of about 1 μm) have been prepared with a pipette puller and one needle has been directly calibrated under liquid: from a procedure based on Stokes' law a stiffness of 1.7 pN/μm ($\pm 20\%$) has been obtained. Other needles are cross-calibrated. Before the force measurements, the needle is coated with biotin, mounted on a xyz micromanipulator, introduced through the free meniscus into the solution and attached to a molecular construction by touching a bead under the microscope. The bead is held a few micrometers above the surface of the glass slide. This is the basic configuration of our micro-needle measurements on the opening of DNA. It is illustrated in Fig. 2. Keeping the base of the micro-needle fixed, we pull on the molecular construction by a slow (typically 20–800 nm/s) piezo-driven, lateral displacement of the slide. This leads to a progressive opening and unwinding of the DNA double helix.

The deflection of the micro-needle as a function of the sample displacement gives the variations in the force occuring during the opening. A numerical analysis of the bead position in the microscope image
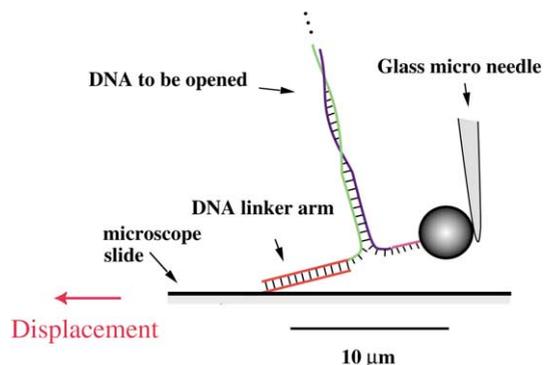
Figure 2. Schematic view of our configuration for opening DNA and force measurements with glass micro-needles.
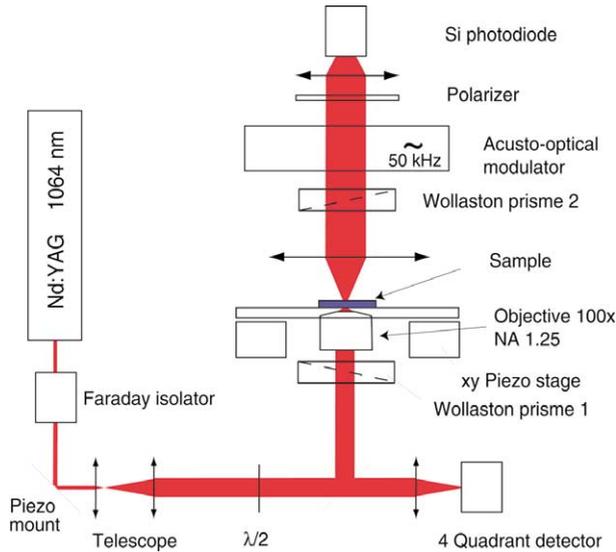
Figure 3. Schematic view of our optical trapping interferometer.

allows us to resolve changes in the deflection down to about 50 nm, which for a typical lever stiffness of 2 pN/μm gives a force resolution of about 100 fN.

Our optical trapping interferometer is inspired by a force measurement setup described by Svoboda et al. [22] which is itself based on a position measurement technique introduced by Denk and Webb [23]. The optics part of our set-up is presented in Fig. 3. The beam of a diode pumped Nd : YAG cw laser (1064 nm) passes a Faraday isolator, is extended by a telescope arrangement, is split with a Wollaston prism and is focussed by an ($100\times$, $NA = 1.25$) oil immersion objective. In the focal plane of the objective two partially overlapping, diffraction limited spots arise with orthogonal polarisation and a center to center distance of about 200 nm. With this arrangement, a silica bead (diameter of 1 μm) is trapped close to the center of the twin focussed spot. This optical trap is well described by a harmonic potential with a stiffness k_{trap}. The transmitted light is collected by a condensor assembly and the two polarisations are recombined using a second Wollaston prism. With a bead captured in the center of the trap the recombined light is linearly polarised. However, a displacement of the bead due to an external force acting along the line connecting the two focal points leads to a difference between the optical path of the two beams and induces an ellipticity in the recombined light. This ellipticity is measured with a lock-in technique using an arrangement involving an acousto-optic modulator, a linear polariser and a silicon photodiode [24]. The analog output signal of the lock-in amplifier passes a frequency adjustable anti-aliasing filter, is converted by an ADC and the numerical data is written to a hard disc. The setup measures sub-nm displacements of the bead with a bandwidth of several kHz. Placed on top of a coarse xy translation stage, a piezo translation table with capacitive position sensors allows to control the relative lateral sample position with nm precision and to impose different computer generated displacement cycles. Finally, in order to suppress small fluctuations of the lateral trap position arising from laser beam pointing, we have introduced a feedback loop in the excitation path which includes a piezo mirror mount and a quadrant photodiode.

We have calibrated the trapping interferometer by two different approaches. In the first case, the power spectrum of a trapped bead is measured [22,25]. The Brownian motion spectrum of a bead in a parabolic trapping potential is of Lorentzian shape and allows us to fit well the measured spectra for all laser powers. The two calibration parameters, the trap stiffness k_{trap} and the ratio between interferometer output and bead displacement increase linearly with the laser power P. With a laser power P of 700 mW, we obtain a stiffness k_{trap} of 250 pN/μm for silica beads of 1 μm diameter in H_2O. The second approach is based on the viscous force acting on a trapped bead when the surrounding liquid moves. With the piezo stage we impose

Figure 4. Schematic view of our configuration for opening DNA and force measurements with an optical trapping interferometer.
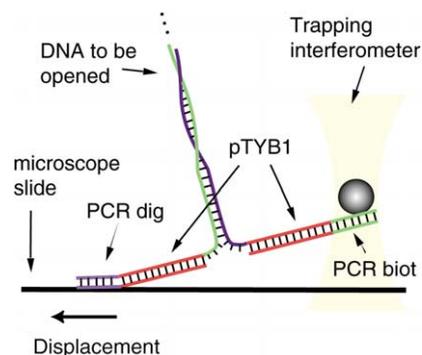
sinusoidal oscillations of various amplitude A_0 and frequency ω_0 and measure the resulting interferometer output. The sinusoidal ouput is phase-shifted by $\pi/2$ with respect to the stage displacement because the force on the bead is proportional to the velocity, as predicted by Stokes law. The output amplitude increases linearly with the amplitude A_0 and with the frequency ω_0 of the oscillation of the stage. This techniques gives a calibration factor (independent of P and with no noticeable difference between the amplitude and the frequency series) which directly relates the interferometer output to the force on the bead, but does not give the trap stiffness. The force calibration obtained by the two different techniques differ by about 10%. We find a non-negligable bead to bead variation of the calibration factor. Performing an oscillation amplitude series on 64 different beads we obtained calibration factors in a $\pm 10\%$ interval around the average value.

For a force measurement, the trapping laser beam is focussed on a bead that has been selected as described above. This bead is held at a height of 3–5 μm above the anti-dig coated surface during the measurement (the height of the objective is measured with an inductive position sensor). This configuration is shown in Fig. 4.

All measurements presented in this paper have been conducted under liquid, in PBS solutions with nearly physiological salt concentrations (pH 7, 10 mM phosphate, 150 mM NaCl).

3. Results

Let us first consider a force measurement performed with a soft micro-needle and with a molecular construction containing a single long linker arm (Fig. 5). After preparation of the measurement as described in the previous section, we laterally displace the sample holder. With increasing displacement, at first the double stranded DNA linker arm is extended to about its contour length, still at a relatively low force F. Afterwards, F rises rather steeply to a value above 10 pN, where the beginning of a quasi-plateau in F is observed. This quasi-plateau extends over more than 40 μm in displacement and corresponds to the successive opening of the Watson–Crick base pairs. At about 65 μm in Fig. 5, all base pairs are opened but the strands do not separate because of the ligated hairpin loop. The final force increase (terminated by a rupture event) thus corresponds to extending a long single stranded DNA molecule. Looking at the signal on the quasi-plateau, we observe that the measured force exhibits a rapid variation which is very rich in detail and has a typical amplitude of 1–2 pN. The overall shape of this force curve closely reflect the G–C versus A–T content of the known sequence of the λ DNA molecule under study. Regions with higher content of G–C base pairs open at higher force than A–T rich regions.

Several measurements have been conducted, both on the same molecular construction by opening and reclosing in repetition and on different constructions with the same genomique sequence. To further substantiate that the force signal is sequence specific, we have prepared a second molecular construction where the DNA to be opened is incorporated in an opposite orientation, which means that the opening fork (position where the two strands separate) runs in the reverse direction through the sequence. The measurements performed on this construction indeed gave the same global features as the measurements
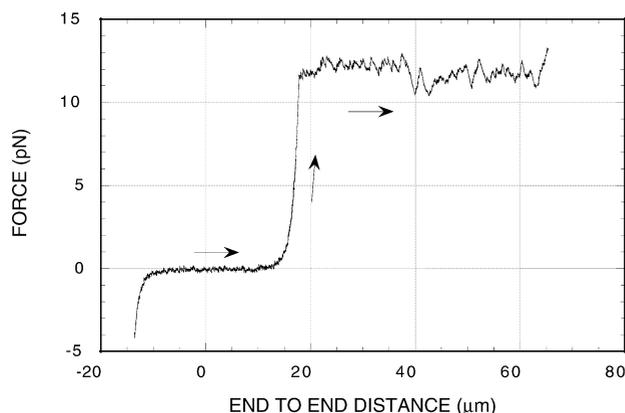
Figure 5. Opening DNA with a soft glass micro-needle. Force (deflection of the calibrated needle) as a function of the end to end distance, measured while pulling the molecular construction with a displacement velocity of 200 nm/s. The end to end distance is defined as the difference between the imposed displacement and the lever deflection. At the origin (0 µm) the needle is positioned directly above the anchoring point of the DNA on the glass slide. A displacement in positive or negative direction leads to a symmetrical deflection of the needle, shown as a positive force, respectively negative force.

performed on the original construction, but in reverse order as expected. This simple relation between force signal and G–C content of the base sequence however only applies to the important features in the G–C content, occuring on a rough scale of about 1000 base pairs.

At a finer scale, the opening of two molecules with identical sequence from opposite sides gives two force signatures which are not simply related by symmetry. Clear saw-tooth like structures appear in the force versus displacement curves, consisting, in the direction of opening, of a slow rise in force followed by a sudden drop in force. This shape is a characteristic feature of stick–slip processes in solid friction (see [26–28] and references therein): during the first phase strain energy is accumulated and in the second phase this energy is transfomed in kinetic energy. A detailed analyis of the opening process, including a theoretical description based on equilibrium statistical physics, indicates that in our measurements the opening fork does not advance continuously under a constant imposed velocity of sample displacement. Depending on the position on the base sequence, a given amount of displacement may induce a very small (stick) or a very large (slip) advancement of the opening fork. A number of fundamental differences exist between this molecular stick–slip process and its classical counterpart. The present molecular process is determined by the sequential exploration of a fixed potential landscape given by the sequence of base pairs. The measured force signals exhibit only a very weak dependence on the velocity of sample displacement and appear to be reasonably well described by a model based on the assumption of thermal equilibrium. The classical stick–slip motion is known to be a strongly velocity dependent, non-equilibrium process.

In Fig. 6, we directly compare the unzipping signal of a micro-needle measurement with the signal obtained with the trapping interferometer. Opening the same sequence of base pairs, the trap data gives a significantly higher density of structure in the force versus displacement curve. The force signal is composed of a succession of sawtooth like structures. For a given additional displacement the opening progresses a little during the gradual rise in force and a lot during the drop in force. As already mentioned this behaviour can be understood in the framework of equilibrium statistical mechanics as an interplay of the complex energy landscape caused by the DNA base sequence, the Brownian motion and the compliances of the molecule and the optical tweezer [7,8].

The stiffness of the trapping interferometer is about two orders of magnitude higher than the stiffness of the glass micro-needle (250 versus 2 pN/µm) and the two ds-linker arms of the new molecular construction are about three times stiffer than the single λ-DNA linker (total length of 14 560 bp versus 48 502 bp). As shown in Fig. 6, the calculation indeed predicts that the resulting increase in total stiffness leads to the experimentally observed gain in base pair sensitivity. In the micro-needle case, the amplitude of the molecular stick–slip motion is stronger. This is exemplified by the presence of more pronounced steps and

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Figure 6. Comparison of a force signal measured with a soft micro-needle (middle) and a signal measured with the trapping interferometer (bottom). Both experimental curves are compared to a calculation based on the assumption of thermal equilibrium. The micro-needle data has been recorded with a displacement velocity of 20 nm/s, the optical trap data with a velocity of 200 nm/s. The two measurements correspond to opening of about the same sequence of the λ-DNA. The calculated average number of opened base pairs ⟨j⟩ as a function of displacement is given in the upper part of the figure.
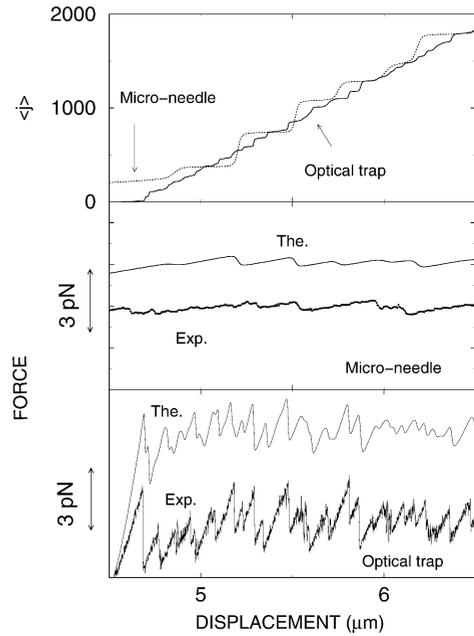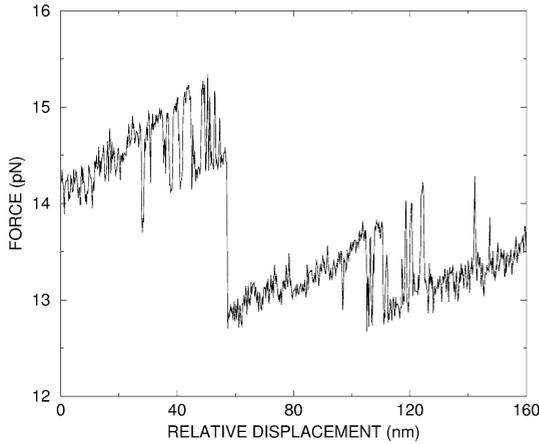
Figure 7. Opening DNA with the trapping interferometer: variation of the force signal with increasing displacement. The measurement (bottom) corresponds to opening with a displacement velocity of 100 nm/s. The upper curve is the result of a calculation based on the assumption of thermal equilibrium. Two intervals of displacement are shown. They roughly correspond to opening from base pair 1 to 2800 (left) and 20 100 to 23 600 (right). The calculated curve is upshifted by 3.3 pN for convenience.
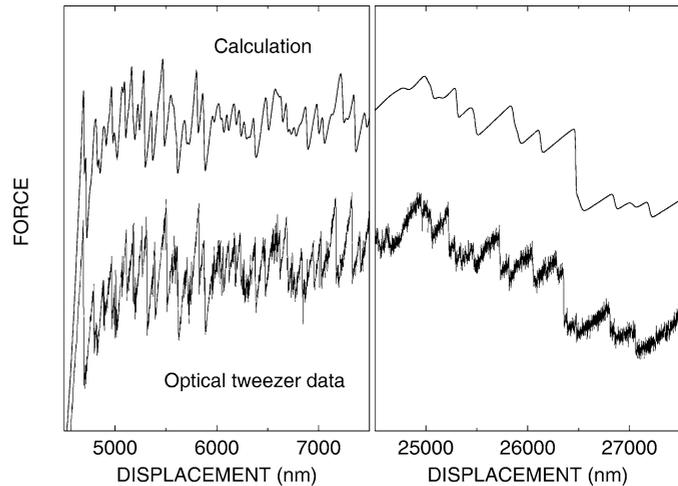
plateaus in the curve describing the progression of the opening fork with increasing displacement (upper part).

In Fig. 7, the force signal of an optical trap measurement is presented, for two different displacement intervals. The experimental data (bottom curves) is compared to a calculation (upper curves, upshifted for presentation purpose).

One way to quantify the sensitivity of the force signal to the base sequence is to consider the calculated variance in the number j of opened base pairs (amplitude of the thermal breathing of the opening fork). This variance var j exhibits a rapid variation as a function of the local sequence. During opening of the first 2000 base pairs (Fig. 7, left part), the stick phases correspond to an amplitude of 5–10 bp, while during the slip events the averaging involves 20–50 bp. If a constant sequence is assumed, we analytically find that

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Figure 8. Opening DNA with the trapping interferometer and with very slow displacement (20 nm/s). Several force flips are observed in the vicinity of the decreasing parts of the sawteeth.

var j is proportional to $k_{\text{tot}}^{-1/2}$. The total local stiffness of the system is given by

$$k_{\text{tot}}^{-1} = k_{\text{ss}}^{-1} + k_{\text{ds}}^{-1} + k_{\text{trap}}^{-1} \qquad (1)$$

with k_{ss}, k_{ds} and k_{trap} being the local stiffness at the opening force of the single stranded DNA, the double stranded DNA and the measurement device, respectively. In the trap measurements, k_{tot} is mainly determined by the molecular compliance, while for the micro-needle measurements both the molecular construction and the force measurement device contribute to the total stiffness k_{tot}. The value of k_{tot} decreases with increasing displacement because the length of the ss-DNA increases. This leads to the systematic decrease in the slope of the rising part of the sawteeth observed in Fig. 7. Concomitant with that, the average size of the sawteeth increases, the density of features decreases and the average noise amplitude increases with increasing sample displacement.

Let us now briefly consider fluctuations occuring in the force signals measured upon opening DNA at very low displacement velocity (below about 200 nm/s). In Fig. 8, a detail of a force curve is presented, where pronounced flips appear in the measured signal. These flips are not predicted by the theoretical description based on the assumption of thermal equilibrium. We observe such bistabilities in the measurements performed with the trapping interferometer but not in the measurements done with soft micro-needles. They also appear only at low displacement velocity and close to the decreasing part of a sawtooth. Under appropriate conditions such flips are frequently observed even without sample displacement.

The physics of this force flipping is considered in detail in [15]. Roughly speaking, our interpretation involves the presence of two local minima in the energy landscape defined by the complex base pair sequence. The two minima are separated by a barrier sufficiently low to allow for spontaneous, thermally induced transitions. One of the two minimum regions corresponds to higher force F and a lower average number $\langle j \rangle$ of opened base pairs, the other one to lower F and higher j. During the rising part of a sawtooth, corresponding to the 'stick phase' of the molecular stick–slip motion, only one minimum region is present in the energy landscape and force flipping is not expected in the corresponding part of the force curve, in accordance with our experimental findings.

A related force bistability has been observed recently by Liphardt et al. [29]. The group has performed optical tweezer measurements on single RNA molecules and has reported flips between folded and unfolded states which correspond to association/dissociation of about 20 RNA base pairs. The amplitude in force and the frequency of the flips in the RNA measurement are comparable to our DNA results.

With the force flips, we have presented a first exemple of a significant difference between the experimental data and the predictions of thermal equilibrium theory. Pronounced deviations are also

Figure 9. Opening and reclosing DNA with the trapping interferometer and with a displacement velocity of 8 μm/s. The displacement is reversed at $t = 3.6$ s. A sizeable, velocity induced shift is observed between the force levels corresponding to the opening part and to the closing part of the measurement.
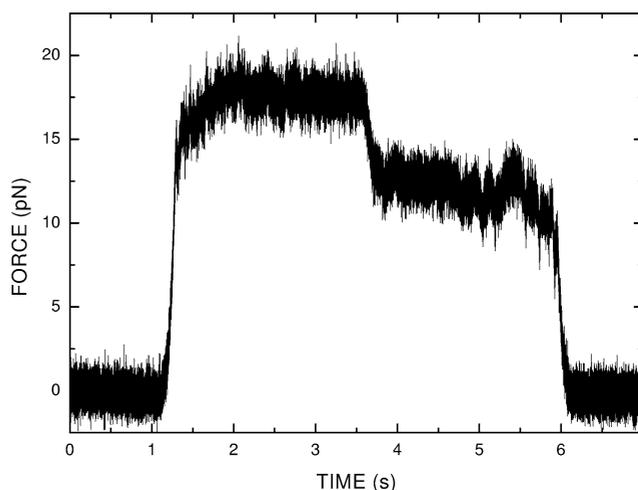
expected at high displacement velocities. In Fig. 9, a force signal is presented as a function of time, for opening and reclosing a molecule with a velocity of 8 μm/s. In this case, unzipping occurs with an average rate of 10 000 base pairs per second and the double helical tail of the molecular construction rotates with about 1000 turns/s in average! The average force level measured during opening is significantly higher than the one measured during closing, while equilibrium theory predicts a perfect symmetry between opening and closing. In our single molecule configuration, we have been able to study rotational drag on double stranded DNA by measuring the force during mechanical opening and closing DNA at different velocities [16]. In the course of the measurement, the DNA double helix is cranked at one end by the effect of unzipping and is free to rotate at the other end. If a sufficiently fast displacement is imposed, the rotational drag on the double stranded DNA leads to a sizeable contribution to the opening force. We find that this contribution increases with increasing velocity and with increasing length of the double stranded DNA.

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