Dynamic force spectroscopy of single DNA molecules

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ABSTRACT To explore the analytic relevance of unbinding force measurements between complementary DNA strands with an atomic force microscope, we measured the forces to mechanically separate a single DNA duplex under physiological conditions by pulling at the opposite 5'-ends as a function of the loading rate (dynamic force spectroscopy). We investigated DNA duplexes with 10, 20, and 30 base pairs with loading rates in the range of 16–4,000 pN/s. Depending on the loading rate and sequence length, the unbinding forces of single duplexes varied from 20 to 50 pN. These unbinding forces are found to scale with the logarithm of the loading rate, which is interpreted in terms of a single energy barrier along the mechanical separation path. The parameters describing the energy landscape, i.e., the distance of the energy barrier to the minimum energy along the separation path and the logarithm of the thermal dissociation rate, are found to be proportional to the number of base pairs of the DNA duplex. These single molecule results allow a quantitative comparison with data from thermodynamic ensemble measurements and a discussion of the analytic applications of unbinding force measurements for DNA.

The direct measurement of forces between individual biological ligand receptor pairs is an emerging field. With the atomic force microscope (AFM), it has been possible to measure unbinding forces between single ligand receptor pairs, which are typically in the pico-Newton range (1–5). These sensitive force measurements can be performed with a high spatial resolution (5). However, the relevance of the force measurements as an (local) analytical tool depends on the understanding of the relationship between the measured forces and thermodynamic data characterizing the complex (6).

The measured unbinding forces are not a fundamental property of a ligand-receptor pair but depend on the loading rate that is applied to the complex: If the load on the complex increases sufficiently slowly, there is time for thermal fluctuations to drive the system over the energy barrier, and the unbinding force will be small (7). A scaling of the force with the logarithm of the loading rate is expected for a single energy barrier along the unbinding path (8) and was found in AFM experiments on unfolding of protein domains (9, 10) and on rupture force measurements of the P-selectin/ligand complex (11). With a different force probe technique, it has been possible to measure the loading rate dependence of the unbinding forces of biotin/(strept)avidin complexes (12). In this system the loading rate dependence of the force is more complicated, indicating that more than one energy barrier is present along the separation path.

The aim of our paper is to present a deeper understanding of the forces that arise if one separates the two strands of a DNA duplex by pulling at both 5'-ends. For this purpose, we have measured the loading rate dependence of the unbinding forces between complementary DNA strands to get information about the energy profile of the separation path. The knowledge of this dependence will allow us to discuss the possibility of analytic applications of unbinding force measurements between complementary strands of DNA. Our aim is to study whether the unbinding force can be predicted from knowledge of the base pair content of the duplex. Therefore, we determined as a first step how the unbinding forces depend on the number of base pairs (bp) of the DNA duplex.

Following the work of Lee et al. (4), we covalently immobilized complementary oligonucleotides with a 5'-SH modification via a cross-linker on the tip of an AFM cantilever and a substrate (schematically shown in Fig. 1A), both modified with aminosilane, respectively (13). We used a 30-nm-long poly(ethylene glycol) (PEG) cross-linker between the oligonucleotides and the surfaces to reduce unspecified forces between the tip and the substrate, which had made the interpretation of previous AFM experiments difficult (4, 14).

The most probable unbinding force can be determined from a histogram of the rupture forces. To facilitate the interpretation of the data, we used oligonucleotides that contain no self-complementary regions and where an “all-or-none” type of binding of the complementary strands is favored. By comparing the forces that arise between the oligonucleotide and itself and the oligonucleotide and its complement, we were able to demonstrate that the measured forces are specific. To investigate the dependence of the unbinding forces on the duplex length, we probed the same single-stranded DNA molecule immobilized on the cantilever tip against complementary DNA strands of different length immobilized on different surfaces. A comparison of the unbinding forces is possible without an exact knowledge of the spring constant of the cantilever. For each duplex, the unbinding force was measured as a function of the loading rate by measuring at different retract velocities of the piezo element where the sample is mounted. The results were interpreted in terms of a single energy barrier along the unbinding path. The rate of dissociation over the barrier (thermal off-rate) was found to decrease exponentially with the number of base pairs in the DNA duplex, allowing a quantitative comparison with thermodynamic data from the literature (15). The separation of the energy minimum from the barrier was found to increase linearly with the number of base pairs. These scaling relations lead to a nonlinear dependence of the unbinding forces on the number of base pairs, which has to be considered when discussing possible analytic applications of force measurements.

METHODS

Surface and Tip Modification. All chemicals were purchased from Fluka unless noted otherwise. The immobilization follows essentially the protocol in ref. 13. Glass slides were cleaned in ethanol for 20 min in an ultrasonic bath and were dried under a stream of argon. From now on, both surfaces, the

The abbreviations used are: AFM, atomic force microscope/microscopy; PEG, poly(ethylene glycol).

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RESULTS AND DISCUSSION

Unbinding Force Measurement. The base sequence of the oligonucleotides we have investigated were designed to favor the binding to its complementary oligonucleotides in the ground state with respect to intermediate duplexes in which the strand is shifted relative to its complement. Therefore, we have chosen the DNA oligomer $a = 5'\text{-G-G-C-T-C-C-C-T-T-C-T-A-C-C-A-C-T-G-A-C-A-T-C-G} \text{-A-G-G-G-A-A-G-A-T-G-G-T-A-C-T-G-T-A-G-C-G-T-G-C-C-S'}$ and the truncated complements $e = 3'\text{-A-T-G-G-T-T-A-C-T-G-T-A-G-C-G-T-T-G-C-C-S'}$ and $d = 3'\text{-T-A-G-C-G-T-T-G-C-C-S'}$ of length 20 and 10, respectively. The duplexes $ab$, $ac$, and $ad$ have approximately the same CG content of 60, 55, and 60%, respectively.

The force spectra between the different oligonucleotides are acquired by approaching the surface to the tip until a repulsive force is sensed. To prevent unspecific binding, the data presented here were recorded under conditions in which this repulsive force on approach never exceeded 150 pN. On retraction of the sample from the tip, the force-displacement curve may show a typical unbinding event like in Fig. 1B. Here, a tip modified with the sequence $a$ is tested against a surface with the complement $b$. The force-displacement curve shows the typical nonlinear elastic behavior of the linker polymer on stretching before the DNA duplex unbinds.

To test the specificity of the measured unbinding forces between the $a$-tip and a complementary $b$-surface, we measured the forces between the $a$-tip and an $a$-surface. The activity of the $a$-surface was afterward proven by testing it against a $b$-tip and the $b$-tip against the previously used $b$-surface. The result of such a measurement is shown in the force histograms in Fig. 2, where the distribution of the last unbinding forces for all four tip-sample combinations is shown. This figure demonstrates that the observed unbinding forces are attributable to the specific interaction of the complementary base pairs.

The forces observed in the measurements of the sequence against itself are very likely caused by unspecific tip-surface interactions, which is indicated by the fact that the unspecific events occur closer to the surface than the specific interaction where both polymeric linkers of 30-nm length have to be stretched before the unbinding of the complex arises. From the
histograms in Fig. 2A, we conclude that not more than 10% of the unbinding forces in the complementary a-tip/b-surface interaction are unspecific.

To quantify the most probable value of the unbinding force for a single complex, one has to work under conditions in which the probability that two or more duplexes unbind at the same time can be neglected. The immobilization of the oligonucleotides via the 30-nm-long PEG linker favors this condition: When two complexes form, it is very unlikely that the two linker molecules, being attached to different points on the tip with a radius of curvature of ≈50 nm, are extended to the same length on stretching. Because of the nonlinear elastic behavior of the linker, the complex with the most extended linker molecule will hold most of the loading force and rupture first. This leads to a situation in which multiple complexes rupture sequentially (Fig. 1C). In the a-tip/b-surface interaction of Fig. 2A, where the total binding probability is ≈75%, 33% of the curves show one rupture event, 20% show two, 15% three, and 7% more than three subsequent rupture events. If the binding obeys Poisson-statistics, one complex would form in 35%, two in 11%, and more than three in 5% of all trials. This excellent agreement with the observed number of rupture events indicates that all multiple binding events have been detected and that it is very unlikely that the last rupture event is attributable to the unbinding of more than one DNA duplex. We found the most probable unbinding force by fitting a Gaussian to the observed rupture force distribution, giving a value of 48 ± 2 pN for the unbinding of the 30 base pairs (with a retract velocity of 50 nm/s) in Fig. 2A. We estimate the statistical error of 2 pN by 2σ/√N (96% confidence level), where σ is the width of the distribution of the N rupture events in the histogram.

**Dynamic Force Spectroscopy.** We measured the most probable unbinding force for the three duplexes ab, ac, and ad in dependence on the retract velocity. For each duplex, typically, 200–400 unbinding events (from 300–600 approach/retract cycles) were recorded at five or six different values of the retract velocity in the range between 8 nm/s and 2,000 nm/s, keeping the approach velocity constant to 100 nm/s. Fig. 3A shows the shift in the unbinding force distribution for the ab duplex by increasing the retract velocity from 8 to 1,600 nm/s. The maximum of the distribution shifts by 20 pN in this case.

![Fig. 2](image1.png)

**Fig. 2.** The probability distribution of the unbinding forces of the last rupture events for ~500 approach/retract cycles for a retract velocity of 100 nm/s. (A) The histograms show the specific a-tip/b-surface interaction (gray) and the unspecific a-tip/a-surface interaction (black). (B) Histograms of the b-tip versus a (gray) and b-surface (black) unbinding force distribution. This tip shows an increase in multiple unbinding forces and unspecific binding. The comparison of all four tip/surface combinations demonstrates the specificity of the unbinding force experiment. In both histograms, the most probable unbinding force is ≈50 pN. The maximum of the distribution is found by a Gaussian fit to be, for example, 48 ± 2 pN in A.

![Fig. 3](image2.png)

**Fig. 3.** Velocity dependence of the unbinding forces. (A) The maximum of the unbinding force distribution for the ab duplex (10 bp) is shifted from 21 to 41 pN (from Gaussian fits) when increasing the retract velocity from 8 to 1,600 nm/s. The broadening of the distribution is partially attributable to an increase of noise in the force displacement curves at higher retract velocities (because the rate of data acquisition is constant, fewer deflection values are acquired to determine the force at higher velocities). (B) The dependence of the most probable unbinding force on the retract velocity for an a-tip against a b-surface (30 bp), c-surface (20 bp), and d-surface (10 bp) and linear fits to the respective data sets. The extrapolation to zero unbinding force gives an estimate for the thermal off-rate.

The results of a measurement, where it was possible to acquire the data for the three duplexes with the same a-tip, is shown in Fig. 3B. Over the range of the measured retract velocities, the data could be interpreted in terms of a cooperative unbinding of the base pairs in the duplex. The cooperative thermal dissociation of short oligonucleotides (17) is described by the thermal off-rate $v = v_0 \exp(-E/k_BT)$, where the frequency $v_0$ is a prefactor, $E$ is the activation energy for dissociation, and $k_BT$ (4.1 pNnm at room temperature) is the thermal energy. In a model with a single energy barrier of height $E$ along the separation path, the application of an external force $f$ leads to an exponential increase of the off-rate $v(f) = v_0 \exp(f/k_BT)$, where the separation $x$ is a length scale describing the separation of the energy minimum from the barrier. If the load on the complex increases with a constant rate $uc$, which is given by the retract velocity $u$ times the elasticity $c$, the most probable unbinding force $F$ depends logarithmically on the loading rate (7):

$$F = \frac{k_BT}{x} \ln \left( \frac{ucx}{k_BT} \right).$$  

[1]
By a linear fit of the data in Fig. 3B, one is able to determine the parameters in Eq. 1 for each DNA duplex; namely, the separation \( x \) from the slope of the linear fit and the thermal off-rate \( \nu \) by extrapolating to zero force \( F \). To calculate the values for the thermal off-rates \( \nu \), the elasticity \( c \) had to be determined from the force-displacement curves: Although the elasticity (force increase per piezo displacement) is a nonlinear function of the force, attributable to the nonlinear entropic elasticity of the PEG linker, the relation in Eq. 1 is still a good approximation if the elasticity \( c \) is chosen to be the elasticity at the most probable unbinding force (18). This elasticity is the slope of the force-displacement curve before the unbinding event occurs, and values from 1.5 to 3 pN/nm for unbinding forces from 20 to 50 pN have been found. The moderate increase of the elasticity with the unbinding forces preserves the linear scaling of the unbinding forces with the logarithm of the retraction velocity to a good approximation. We have adopted a constant value of 2 pN/nm for the elasticity to calculate a numeric value for the thermal off-rate. The values for the data in Fig. 3B and an additional data set (obtained with different tips and surfaces) are compiled in Fig. 4, where the errors reported are the standard deviations from the linear regression.

The data in Fig. 4 indicate clearly that the values for the separation \( x \) and the thermal off-rate \( \nu \) are well reproduced within the statistical error for different data sets. The most important fact to understand the unbinding forces that arise between the complementary oligonucleotides is that both the separation \( x \) and the logarithm of the thermal off-rate \( \nu \) scale in a first approximation linearly with the number of base pairs in the duplex.

**Thermal Off-Rates.** Measurements of the thermal dissociation rates (thermal off-rates) of the investigated duplexes with other methods are not available, but a similar scaling of the thermal off-rate with the number of base pairs \( n \) has been found for oligo(A)-oligo(U) duplexes of 8–18 bp with a temperature jump method (18, 19). An exponential decrease of the thermal off-rate with the number of base pairs is expected because of the increase of the activation energy \( E \) for dissociation (\( \approx 10 \) kT per A-U base pair). However, the frequency prefactor \( \nu_0 \) also strongly increases with the number of base pairs because of the increasing number of degrees of freedom of the system, so that the thermal off-rate was found to decrease approximately as \( 10^{-0.5n} \) s\(^{-1} \) at room temperature (17). Our measured off-rates also can be described by

\[
\nu = 10^{3 \pm 1} \nu_0 s^{-1}
\]

where we found \( \alpha = 3 \pm 1 \) and \( \beta = 0.5 \pm 0.1 \) from a linear regression of the data in Fig. 4A. The fact that the off-rates are lower for duplexes containing CG pairs is known (17), but literature values for CG-rich systems are rare (15). For instance, the thermal off-rate for the duplex (5'-G-G-G-C-3')(3'-C-C-C-G-5') was found to be 40 s\(^{-1} \) (15) at room temperature compared with \( \approx 10^{-1} \) s\(^{-1} \) (calculated on the basis of Eq. 2 with \( n = 4 \)). This suggests a reasonable agreement of our measured off-rates with thermodynamic data. To determine the height of the energy barrier, temperature-dependent off-rate measurements would be necessary.

**Forced Unbinding Pathway.** The length scale \( x \) describes the difference in 5'-end to 3'-end separation between the bound state representing the energy minimum on the one hand and the state representing the energy barrier, i.e., the transition state, on the other hand. To estimate the order of magnitude of the separation \( x \), it is reasonable to assume that the transition states along the mechanical unbinding path are similar to the transition states of the thermal dissociation path: Without applied load, the thermal fluctuations will open the duplex at the helix ends (17), and dissociation occurs if all base pairs are zipped open. Thus, the transition states can be described by a situation in which only a few base pairs are formed. An upper limit for the separation \( x \) can be estimated if we assume that the bound state is a B-DNA helix with an axial rise of 3.4 Å per base pair (20). If the transition state consists only of one base pair and totally stretched single-stranded DNA ends with a distance of \( \approx 7 \) Å between adjacent phosphate groups (20), the separation \( x \) is proportional to the number of base pairs (Fig. 5A), increasing by \( \approx 3.6 \) Å per base pair. A linear regression of the data in Fig. 4B gives

\[
x = (7 \pm 3) \text{ Å} + (0.7 \pm 0.3)n \text{ Å}
\]

for the increase in separation. The increase in 5'-end to 3'-end separation of \( \approx 1 \) Å per base pair is significantly smaller than the maximal expected separation increase of 3.6 Å. This may originate from the fact that the extension of the duplex in the bound (double-stranded DNA) and at the transition state (single-stranded DNA) is not described correctly in the above argument. For instance, the duplex deforms under an applied load, and, for a large (>1,000 bp) duplex, a cooperative transition at 60–70 pN from a B-DNA to a so-called over-stretched S-DNA (B-S transition) is found, where the difference in extension between single- and double-stranded DNA almost vanishes (21, 22). However, the elasticity of the duplex itself cannot be extracted from the force displacement curves directly because the elasticity is dominated by the PEG linker. Certainly, more sophisticated models are needed to understand the 5'-end to 3'-end separation of a DNA duplex at a transition state and to clarify the significance of the offset length of 7 Å in the linear regression (Eq. 3) that accounts for a length scale in the unbinding pathway that is independent of the number of base pairs.

**Comparison with Mechanical Properties of DNA.** We first note that, because of the above relations (Eqs. 1, 2, and 3), the
forces in the range of 50–60 and 70–80 pN for the data of Fig. 2 may indicate the B-S transition of the 30-bp duplex.

The 5'-end to 5'-end unbinding forces measured with our setup also can be compared with experiments in which λ-phage DNA (24) or poly(dG-dC) and poly(dA-dT) hairpins (23) were mechanically separated with a different geometry of the acting forces, namely by pulling apart the 3'- and 5'-end of the two DNA strands. Forces of 11–14 pN for λ-phage DNA, 20 pN for a poly(dG-dC), and 9 pN for a poly(dA-dT) hairpin are needed to separate the two strands, independent of the loading rate and number of base pairs. In this case, the forces are interpreted as the average energy (over ~50 bp) per base pair divided by the 5'-end to 3'-end separation increase per open base pair. One can apply this argument to our 5'-end to 5'-end geometry of the applied force by noting that the 5'-end to 3'-end separation increase is ~2X the 5'-end to 5'-end separation increase per opened base pair (Fig. 5 C and D) if the stretching of the DNA duplex on pulling at the opposite 5'-ends is neglected. This would yield forces 2X bigger, e.g., 18–40 pN when separating a long duplex.

**Analytic Application of Force Measurements.** If one thinks of the detection of hybridization by AFM force measurements (25), e.g., in a DNA sequencing application, the accuracy of the information about the thermal off-rate and the number of base pairs in a duplex is determined by the range of loading rates and the quality of the statistics of unbinding forces at each loading rate. Neglecting a systematic error in the spring constant of 20%, the error in the separation x is already low enough to conclude that one should be able to determine a length difference of a single base pair in a 10-bp duplex. For example, the difference in the most probable unbinding force between a 9- and 10-bp duplex at a loading rate of 300 pN/s should be 3 pN, which is still measurable. Because one mismatched base pair can increase the thermal off-rate by one order of magnitude (26), this should lead to a difference of 7 pN in the unbinding force of a 10-bp duplex at 300 pN/s loading. With the same argument, variations in the CG content should be detectable if they lead to changes in the thermal off-rate by one order of magnitude. For larger duplex sizes, the resolution decreases so that the best contrast in the force is reached for small duplex sizes. Note, however, that, for small duplexes (<5 bp), the thermal off-rate gets too fast to detect an unbinding event in the time scale of the AFM experiment; i.e., the measurement between an a-tip and a surface with the oligonucleotide 3'-T-T-G-C-C-5' showed no unbinding events.

**Conclusion.** The cooperative unbinding of the base pairs in the DNA duplex leads to a scaling of the unbinding forces with the logarithm of the loading rate. This behavior is described with one length scale (the separation x) and one time scale (given by the thermal off-rate). The separation x was found to increase with the number of base pairs, again indicating the cooperativity of the unbinding event. Especially the exponential decrease of the thermal off-rate with the number of base pairs is in good agreement with thermodynamic data. We are thus able to predict values of the unbinding forces from the knowledge of the thermal off-rate of a DNA duplex.

On a molecular scale, the forces between complementary DNA strands can be considered as a model system for ligand-receptor systems in general. Because length scales for separation and thermal off-rates are of a comparable order of magnitude in these systems, our considerations about the analytic application of force measurements can be generalized to these systems.

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