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Discriminating small molecule DNA binding modes by single molecule force spectroscopy

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Abstract Drugs may interact with double stranded DNA via a variety of binding modes, each mode giving rise to a specific pharmacological function. Here we demonstrate the ability of single molecule force spectroscopy to discriminate between different interaction modes by measuring the mechanical properties of DNA and their modulation upon the binding of small molecules. Due to the unique topology of double stranded DNA and due to its base pair stacking pattern, DNA undergoes several well-characterised structural transitions upon stretching. We show that small molecule binding markedly affects these transitions in ways characteristic to the binding mode and that these effects can be detected at the level of an individual molecule. The minor groove binder berenil, the crosslinker cisplatin and the intercalator ethidium bromide are compared.

Key words: Force spectroscopy; Single molecule; DNA; Small molecule binding; Cisplatin; Berenil; Ethidium bromide

1. Introduction

Cell replication and gene expression are regulated through a number of specific DNA–protein interactions. Small molecules may mimic or block these processes and thus offer potential therapeutic agents [1–5]. Critical to the understanding of the function of such molecules and thus also for the development of novel drugs is the characterisation of their binding modes. For example, they may bind through intercalation, groove binding or covalent attachment. In this study we investigate the influence of three well-characterised agents on the mechanical properties of DNA. Specifically, we utilise the crosslinking anti-cancer drug, cisplatin [6,7], the anti-trypanosomal minor groove binder, berenil [8] and the intercalating dye, ethidium bromide [9].

In conventional assays molecular interactions are investigated in ensemble measurements, which by their very nature average over different species and require sizeable amounts of reagents. However, with the growing number of ultrasensitive techniques, measurements at the level of single molecules have become possible, allowing discrimination between individuals. In particular, mechanical experiments with individual molecules, where the extensibility of the selected molecule is measured as a function of the applied force, have provided a wealth of detailed information on the internal organisation of biopolymers and molecular interactions. Such single molecule force spectroscopy investigations have also provided new and direct insight into the structure and function of DNA [10–12].

In solution DNA occurs as an entropic coil. If extended between an AFM-tip and a surface, the molecule exerts a restoring force due to the reduction in degrees of freedom (Fig. 1, black curve). Up to forces of approximately 50 pN the force–extension characteristics can adequately be described by the worm-like chain model [10]. At forces of 65–70 pN the molecule undergoes a highly cooperative structural transition observed as a plateau in the force–extension profile. In this plateau B-DNA can be overstretched to about 1.8 times its contour length [13,14]. Different experimental and theoretical studies have attributed the occurrence of the plateau to a structural transition from B-DNA to an overstretched structure, termed S-DNA, in which most of the stacking interaction of DNA bases is lost. A recent study explains the plateau in the force–extension curves by a mere melting of the double helix [15,16], implying also the breakage of hydrogen bonds in the overstretching transition. During this overstretching transition the strands of the DNA molecule have to rotate in order to alleviate torsional strain, which is possible due to the presence of a nick in one of the DNA strands. Once the molecule has reached the end of the plateau it can be further stretched, and at typical pulling rates of several μm/s another transition is observed at forces around 150 pN [17]. After stretching beyond this melting transition the double stranded molecule is finally separated into two single strands, one of which remains tethered between tip and surface. Subsequently, the relaxation trace (Fig. 1, grey curve) does not resemble the extension trace and single stranded DNA mechanical properties prevail. At lower forces (below 150 pN), partial melting of the DNA molecule can occur and is observable as a deviation of the relaxation trace from the stretching traces (melting hysteresis). The hysteresis between extension and relaxation traces is thus a good indication of a 'force-induced melting'. It should also be noted that melting hysteresis may occur when the molecule is over-

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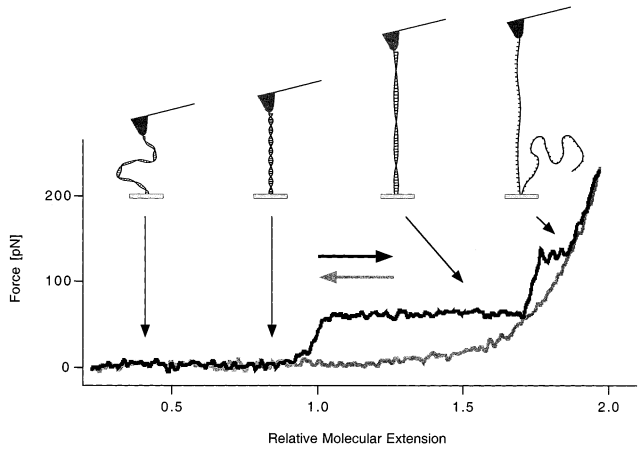

Fig. 1. Schematic of the force–extension characteristics of DNA: at 65 pN the molecule is overstretched to about 1.7 times its contour length, at 150 pN the double strand is separated into two single strands, one of which remains attached between tip and surface.

stretched into the plateau regime and then relaxed. Complete and permanent separation of the double strand, however, is only observed after overstretching the molecule beyond the melting transition. The exact pathway of melting also depends on the exact geometry of the experiment, such as the number and location of nicks on the molecule, and kinetic effects. Therefore, the rate dependent high force melting transition, which is a non-equilibrium process on the time scale of the experiment, cannot be explained by an equilibrium thermodynamic model as in [15,16]. This model, however, predicts that the overstretching force should be related to the thermal melting temperature and that the force range over which overstretching occurs should be related to the width of the thermal melting transition.

In this study we utilised the tip of an AFM cantilever to pick up a single molecule of digested λ phage DNA of mixed AT/GC composition from the sample surface. We characterised this DNA molecule thoroughly by recording several extension cycles. Upon the addition of DNA binding agent to the experimental buffer alterations in the extensibility were then investigated. In particular, any changes in the overstretching transition and the high force transition on the same molecule were examined.

2. Materials and methods

λ phage *Bst*EII-digested DNA, cisplatin (*cis*-platinum(ii)diammine dichloride), ethidium bromide and berenil were purchased from Sigma (Deisenhofen, Germany). Details of the force experiments and sample preparation are described elsewhere [17,18]. For the data shown here single DNA molecules were stretched between AFM-tips and gold substrates. Whilst maintaining a single molecule tethered between the probe and substrate, the experimental buffer in the sample volume (100 μl) was then exchanged by flushing it with approximately 500 μl of drug containing buffer.

For complexation with cisplatin a 2130 nm long fragment of *Bst*EII-digested λ phage DNA (approximately 6260 bp, 50% GC-content) was stretched immediately after adding 50 μl of a saturated solution to the sample volume. The same molecule could then be repeatedly stretched and the reaction followed over a period of 1 h. For the reaction with berenil a 2000 nm long fragment of λ phage *Bst*EII-digested DNA (approximately 5880 bp, 50% GC-content) and berenil concentrations of 1.5 μg ml^{-1} and 15 μg ml^{-1} were used. The reaction with ethidium was followed on a 1200 nm long fragment of λ phage *Bst*EII-digested DNA (approximately 3500 bp, 40–60% GC-content) using concentrations of 0.44 μg ml^{-1} and 2.2 μg ml^{-1} ethidium bromide.

Experiments on other fragments of *Bst*EII-digested λ phage DNA with different length and GC-content, measured over a number of different experiments, showed no qualitative difference in the observed effects. All experiments were carried out with a custom built AFM, in 10 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 1 mM EDTA and the respective concentrations of drug. The indicated ratios of drug molecules per base pair given in the captions refer to the amount of drug in the sample volume and the amount of DNA on the sample after rinsing. The spring constants of all cantilevers (Microlevers, Park Scientific Instruments, Sunnyvale, CA, USA) were determined using the thermal noise method [19]. All displayed force curves consist of 4096 points and were smoothed using a 21 point box integrator. The length of the molecules was normalised to the contour length of each uncomplexed molecule.

3. Results

Previous experiments have shown that the mechanical properties of dsDNA are markedly affected by sequence specific crosslinking with cisplatin [18]. However, it should be noted that the binding kinetics of this process are slow (see Section 4). Fig. 2a shows the force vs. extension curves obtained on a single molecule of DNA immediately after cisplatin was added to the solution. It exhibits the well-known characteristics of native dsDNA. After a reaction time of 1 h – referring to a small amount of cisplatin bound to the molecule – hysteresis between stretching and relaxation is inhibited (Fig. 2b). Additionally, both, the onset and the end of the overstretching transition are shifted towards a lower extension, characteristic of a shortening in the contour length of the molecule. However, since only a minor fraction of the bases have reacted this

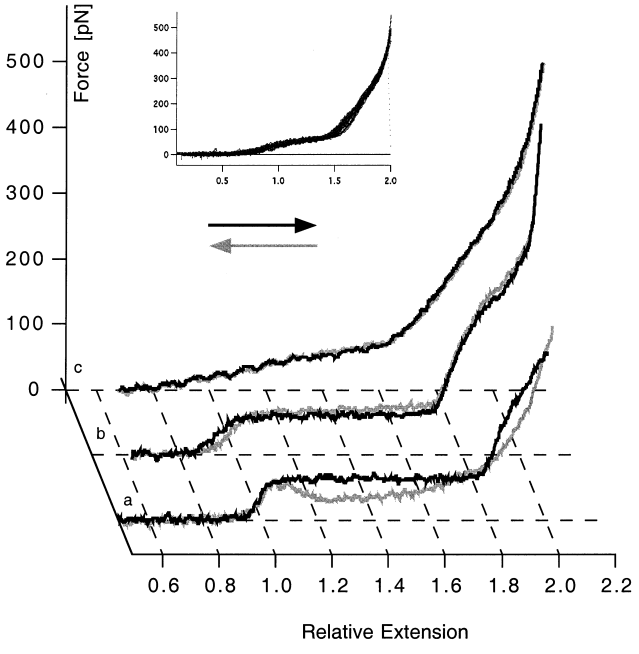

Fig. 2. A single molecule of DNA is stretched between an AFM-tip and a substrate immediately after adding an excess of cisplatin (a). The progress of the chemical reaction is then followed on the same molecule: after 1 h (b) the molecule is shortened and the hysteresis between stretching and relaxation has vanished. After 24 h of reaction (c) the molecule is saturated with cisplatin, resulting in a less cooperative overstretching transition that ends at 73 pN and further shortening of the molecule is seen (cf. [18]). The inset shows the superposition of 30 curves obtained on different molecules.

shortening is only small. For the molecule shown in Fig. 2, the extension to the end of the plateau was shortened by 3.5%. The force of the overstretching plateau remains at ca. 65 pN and is thus unchanged within the error of the measurement. After a reaction time of 24 h – and hence more cisplatin bound to the molecule – the overstretching plateau yielded to a steady rise in force up to 73 pN, indicating a large reduction in the cooperativity of the transition (Fig. 2c, obtained on a different molecule of the same kind). Even when the molecule was kept under tension for several minutes no hysteresis between stretching and relaxation in the following force–extension cycle was observed. All displayed curves (and all curves in Figs. 3 and 4) are typical examples of individual single molecule stretching cycles. They could be superimposed with many other curves obtained in various experiments on molecules of different length, when scaled to the contour length of the molecule. The inset in Fig. 2 shows the superposition of 30 curves obtained on different molecules with pulling velocities between 0.1 μm/s and 5 μm/s.

Ethidium bromide is a well-characterised dye that intercalates into DNA without sequence specificity. Insertion of a single dye molecule increases the base pair base pair rise by 3.4 Å and unwinds the double helix by 26° [20]. Typical force extension traces obtained on DNA in the presence of ethidium bromide show that low concentrations (0.44 μg ml^{-1} or approximately 1 molecule of ethidium per 10 bp) (Fig. 3b) markedly affect the overstretching plateau. The increased slope of the transition regime is indicative of a reduction in cooperativity as compared to the curve obtained on the very same molecule before ethidium was added (Fig. 3a). The force is reduced in the beginning of the transition while at the end of the transition it rises above 110 pN, almost twice the force at

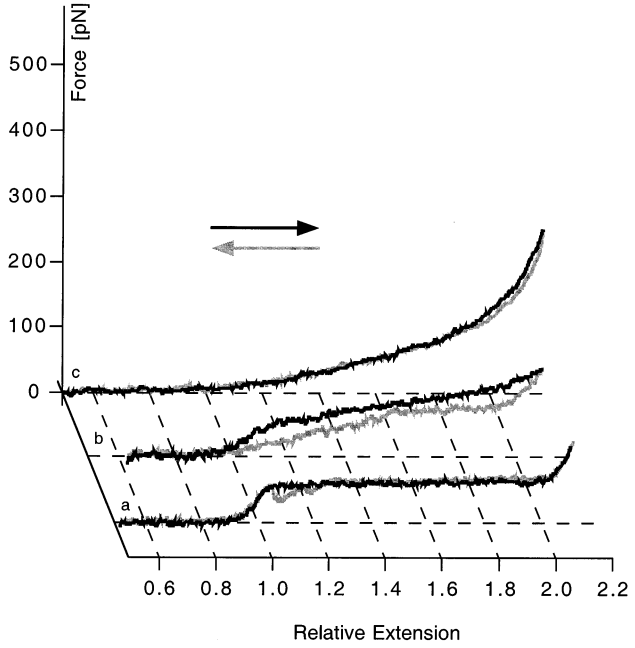

Fig. 3. Force vs. extension curve of a single molecule of DNA in pure buffer (a) and in the presence of 0.44 μg ml^{-1} (b) and 2.2 μg ml^{-1} (c) ethidium bromide, referring to approximately 1 molecule of ethidium bromide per 10 and 2 bp, respectively. With increasing concentration of ethidium bromide the overstretching plateau shortens while the force at the end of the transition increases to 110 pN. At high concentrations the hysteresis between stretching and relaxation is drastically reduced.

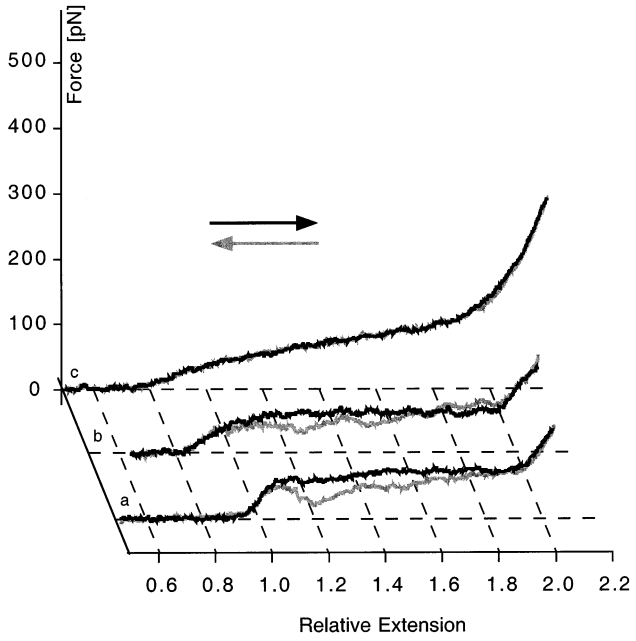

Fig. 4. A single molecule of DNA is stretched in pure buffer (a). After berenil is added in a concentration of 1.5 μg ml^{-1} (approximately 1 molecule per 4 bp) the force curve only deviates from the previous curves in the low force regime, indicating a minor deviation from the native conformation (b). Increasing the concentration of drug to 15 μg ml^{-1} (approximately 1 molecule of berenil per 0.4 bp) results in a drastic change in the force curve: hysteresis is reduced and the force at the end of the transition is increased to 95 pN (c).

the end of the overstretching transition in non-complexed molecules. The melting transition, however, is still observed and hysteresis between stretching and relaxation remains. At elevated concentrations (Fig. 3c), the plateau vanishes and a steadily rising force is observed that begins at larger extensions. Furthermore, the sharpness of the end of the overstretching plateau is lost, and it is difficult to precisely identify the beginning or end of the overstretching transition. Additionally, at this concentration no distinct melting transition and very little hysteresis between stretching and relaxation is observed, indicating either an accelerated reannealing process or the prevention of force-induced melting. While it is not possible to define a transition force in the presence of higher concentrations of ethidium, the curves obtained in the presence of small amounts of ethidium show that overstretching occurs at higher transition forces and over a wider force range (approximately 50 pN) as compared to untreated molecules.

Berenil is a well-established minor groove binder, which binds selectively to the narrow minor groove of AT rich regions. Binding is mediated through hydrogen bonding via the bis-amidinium groups at each end of the molecule, and through van der Waals interactions within the minor groove. Generally minor groove binding has negligible influence on the B conformation of DNA, however at higher concentrations of berenil an intercalative binding mode has been proposed [8].

Typical force extension traces obtained on DNA in the presence of 1.5 μg ml^{-1} berenil (approximately 1 molecule per 4 bp) show that the extension characteristics are altered only in the low force regime; the onset of the overstretching transition is broader and shifted to shorter lengths (Fig. 4b).

Stiffening of the molecule, which is conceivable on binding of the berenil would result in deviation from the standard dsDNA curve. From this point onwards the force–extension curve resembles that of non-complexed DNA, and force-induced melting is still observed. At higher concentrations of 15 μg ml^{-1}, referring to approximately 1 molecule of berenil per 0.4 bp, the overstretching transition has lost more of its cooperativity and a distinct plateau is no longer observed (Fig. 4c). This is again replaced by a steadily rising force, ending at approximately 95 pN. At these concentrations a melting transition is infrequently seen in the force–extension curves, however, no hysteresis is observed. This lack of hysteresis following melting suggests that the two molten single DNA strands are maintained within a close proximity of each other and can reanneal quickly.

4. Discussion

The observed changes in the stretching curves were found to be characteristic for each DNA binding agent. Furthermore, they were found to be very reproducible and not dependent on the force to which the molecules are stretched. It should be noted, however, that relaxation traces are more individual for each molecule since they include the effects of reannealing of the molten strands. The rate of reannealing does largely depend on the presence and position of nicks in the two DNA strands and therefore differs between different molecules. Despite this, the observed changes for an individual molecule of DNA upon binding of a certain agent were found to be characteristic for the effects of each drug.

Binding of cisplatin to DNA is known to occur slowly and to involve initial monofunctional binding of cisplatin followed by the formation of bifunctional crosslinks [6]. Therefore, only a small number of crosslinks will have formed within 1 h of reaction time. This however, is easily detected in the force extension characteristic of the DNA molecule, resulting in a shortening of the molecule and the complete loss of melting hysteresis. The complete loss of melting hysteresis between stretch and relaxation after complexation with cisplatin indicates that the molten single strands are able to recombine more quickly due to the formation of drug–DNA crosslinks. Such crosslinks keep the two strands in close proximity, giving rise to accelerated reannealing without markedly altering the extensibility. Cisplatin increases the force range over which overstretching occurs, indicating reduced cooperativity of the overstretching process. The slow increase in force during the overstretching can also be interpreted as a lowered overstretching force. As predicted by [15,16] this agrees with the reported lower melting temperatures and wider temperature range in thermal melting experiments [21,22].

For ethidium bromide binding the shortened transition reflects the pre-stretching and unwinding of the DNA by intercalation, while the higher force at the end of the transition indicates a structure which is stabilised as compared to the non-complexed molecules. Thermal melting experiments showed an increase in melting temperature for ethidium-intercalated DNA with a wider range of melting temperature (data not shown and [22,23]). As for cisplatin, the melting temperature and range correspond to the observed changes in force and force range of the overstretching transition.

At low concentrations berenil is found to change the extension characteristics of DNA only in the low force regime below 70 pN. This is conceivable when the effects of minor groove binding, which involve only minor changes in the B-DNA structure, are considered. The lack of effect in the high force regime may be explained by the fact that the affinity of berenil to DNA is largely dependent on the local structure of the double helix. Stress-induced changes in local structure, and in particular a change in the width of the minor groove, may therefore also result in an altered mode of interaction of the drug with the DNA. The drastic change in the shape of the force curve at high concentrations of berenil cannot be explained by mere groove binding. However, such alterations in behaviour are conceivable when an additional intercalative binding mode is considered. A comparison of typical stretching curves obtained in the presence of higher concentrations of berenil to those obtained at a low concentration of ethidium bromide also reveals great qualitative similarity. Ethidium bromide binding, however, seems to have a stronger stabilising effect on the mechanics, resulting in higher forces.

Following berenil binding, the force range of overstretching increases to approximately 50 pN, indicating reduced cooperativity of the overstretching process. The overstretching force, however, remains constant within the error of the measurement (± 5 pN). Thermal melting studies showed that in the presence of berenil, DNA denatures at higher temperatures (own data not shown and [24–26]). These studies have also shown that the temperature range over which melting occurs is dependent upon DNA sequence. While the melting was found to occur over a smaller temperature range (and hence more cooperatively) for most molecules, the temperature range actually increased for poly(dGdC)•poly(dGdC). The variation in such results makes it difficult to explain the melting behaviour of mixed sequences of DNA, as observed in our experiments, by a simple two state thermodynamic model.

Recent theoretical work suggests that the cooperativity of the overstretching transition is strongly dependent on the base stacking in the DNA double helix [27]. It is therefore conceivable that different perturbations of the stacking interaction could give rise to unique force curve profiles. Indeed, different binding modes of small molecules are known to cause different perturbations in base stacking [1,2,5–8] and may therefore be discriminated by the respective force vs. extension data.

In all of our experiments the measured extensibility profiles of the DNA were found to be highly characteristic of the respective binding mode of the agents tested and also revealed a strong concentration dependence. Improved theoretical models and expanded investigation of the sequence and concentration dependence of the observed effects will help to further correlate the force curves to the structural changes induced by small molecule binding. This will also help to clarify the exact contributions of stacking and hydrogen bonding interactions to the force extension characteristics. Force spectroscopy thus promises to become a helpful and sensitive tool for the investigation of DNA-drug binding modes on the single molecule level. For a broad application of this novel technique, for example for screening purposes, a parallelisation of the technology will be essential and with several different labs and companies working in parallel towards this goal there is justified hope.

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