Mapping the Phase Diagram of Single DNA Molecule Force-Induced Melting in the Presence of Ethidium

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When a single DNA molecule is stretched beyond its normal contour length, a force-induced melting transition is observed. Ethidium binding increases the DNA contour length, decreases the elongation upon melting, and increases the DNA melting force in a manner that is consistent with the ethidium-induced changes in duplex DNA stability known from thermal melting studies. The DNA stretching curves map out a phase diagram and critical point in the force-extension-ethidium concentration space. Intercalation occurs between alternate base pairs at low forces and between every base pair at high forces.

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The interaction of small molecules, or drugs, with DNA has been investigated due to its implications in rational drug design for cancer therapy. Recently, single molecule manipulation techniques [1–4] have been used to probe drug-DNA complex formation. In this work, we use optical tweezers to systematically study the effect of ethidium binding on the stretching behavior of single DNA molecules. We estimate the drug's effect on DNA duplex stability, and evaluate its DNA binding parameters. We then obtain a complete characterization of the thermodynamics governing DNA intercalation with and without stretching forces.

Ethidium, obtained upon addition of ethidium bromide (EtBr) to solution, is considered a paradigm for intercalative binding to double-stranded DNA (dsDNA) [5,6]. In intercalative binding, a flat, planar molecule is inserted between successive bases on a DNA molecule, increasing its length. By studying the effect of ethidium intercalation on the DNA force-induced melting transition (Fig. 1), we determine the increase in DNA melting free energy due to ethidium binding, or the free energy required to simultaneously melt the DNA and unbind the drug, and find agreement with bulk measurements of this free energy. This, together with the behavior of the hysteresis between stretching and relaxation curves obtained in the presence of ethidium, which is only observed upon stretching into the transition region, suggests that this constant force transition represents the conversion of ethidium-bound dsDNA into single-stranded DNA (ssDNA). In addition, we show that under strong stretching forces, ethidium is capable of binding to every base stack, in violation of the "site exclusion principle" previously proposed [7].

The present study also allows us to obtain a complete phase diagram of the ethidium-DNA melting transition in the 3-dimensional (force-extension-drug concentration) space. We find a critical concentration $[\mathrm{EtBr}]_{cr}$ at which the coexistence region of ethidium-dsDNA with ssDNA vanishes, such that at higher [EtBr] the phase separation becomes impossible, and no force-induced DNA melting occurs. We draw a direct analogy between the phase transition in our single DNA molecule and the conventional liquid-gas equilibrium.

For these studies, we investigate single DNA molecule-ethidium interactions using an optical tweezers instrument, which was previously described [8–10]. Briefly, two laser beams are focused to a small spot, creating an optical trap that attracts polystyrene beads. Single DNA molecules are attached at one end to a bead in the trap, while the other end is attached to a glass micropipette. As the torsionally unconstrained DNA molecule is stretched by moving the micropipette, the resulting force on the bead in the trap is measured. Force-extension curves for a single DNA molecule in the presence of different EtBr concentrations (in 10 mM HEPES, 100 mM NaCl, *p*H 7.5, room temperature) are recorded.

In the absence of drug, we obtain a constant force plateau upon stretching that extends from 0.34 to 0.58 nm/bp,

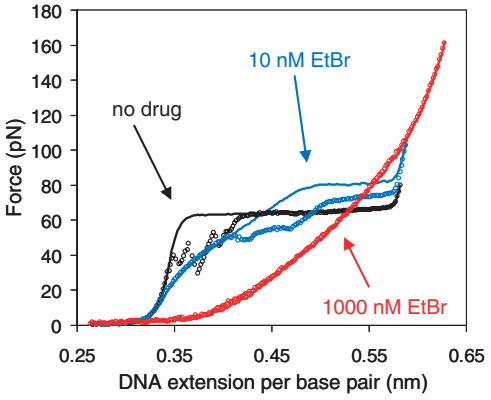

FIG. 1 (color). DNA stretching (solid line)—relaxation (circles) cycles of pure DNA and the DNA-ethidium complex. At low EtBr concentration, the DNA melting transition is still present.

as shown in Fig. 1. The relaxation curve follows the original stretching curve until it reaches 0.4 nm/bp, where the two curves exhibit hysteresis. This hysteresis has been attributed to the inability of the two melted DNA strands to reanneal on the time scale of the relaxation [11]. We obtain two different types of stretching-relaxation cycles in the presence of low and high concentrations of EtBr (Fig. 1). At concentrations less than $[\mathrm{EtBr}]_{\mathrm{cr}}$, the DNA stretching curves exhibit a cooperative force-induced transition similar to that seen in the absence of the drug. Further DNA extension beyond the plateau region results in a steep force increase, similar to that which occurs in the absence of drug, independently of added ethidium. This rise in force resembles the stretching behavior of single-stranded DNA [1]. The DNA relaxation curves show hysteresis only when the DNA is stretched into or beyond the plateau region. At drug concentrations higher than $[\mathrm{EtBr}]_{\mathrm{cr}}$, the force-extension curves are simple, monotonically increasing functions, which shift to longer extensions until the length increase saturates (Fig. 2). No relaxation hysteresis is ever observed at drug concentration greater than $[\mathrm{EtBr}]_{\mathrm{cr}}$. Thus, ethidium binding to DNA is always in equilibrium with respect to much slower conformational changes in the DNA.

All of the DNA stretching features described above can be consistently interpreted by assuming that the constant force transition observed in the absence of drug and at low drug concentrations corresponds to a force-induced DNA melting transition, in which the base pairs that form the DNA helix are broken [11]. Ethidium-DNA binding studies have shown that this drug binds much stronger to dsDNA than to ssDNA, thus stabilizing the DNA duplex. In our force-extension curves this EtBr-induced duplex stabilization results in an increase in the force F_m at the force-induced elongation plateau. This plateau corresponds to a highly cooperative melting transition of the entire ethidium-DNA complex, which shortens due to the progressively increasing length of the ethidium-dsDNA complex.

To quantitatively test this hypothesis, we determine the melting transition free energy, $\Delta G(\mathrm{EtBr}\text{-DNA})$, as a function of [EtBr] from the area of the mechanical cycle in which the DNA is stretched as ethidium-dsDNA, and released as a ethidium-free ssDNA:

$$\Delta G(\mathrm{EtBr\text{-}DNA}) = \int_0^{F_m} df\Big(x_{ss}(F) - x_{ds-\mathrm{EtBr}}(F)\Big), \quad (1)$$

where $x_{ds-\mathrm{EtBr}}(F)$ is the length of the ethidium-dsDNA complex corresponding to the stretching force F and $x_{ss}(F)$ is the length of the ssDNA molecules under tension. When the dsDNA melts, it converts to alternating regions of one or two single strands under the same tension, depending on the number of breaks present in the DNA backbone [12], and this may result in up to 30% error in our estimate of the melting free energy. However, measurements of the change in ΔG with [EtBr], $\delta\Delta G(\mathrm{EtBr})$, obtained on the same molecule, should have less than 10% error. In Fig. 3, the result of the latter calculation is compared to the change in free energy obtained from the published data on the dependence of the polymeric DNA melting temperature T_m on [EtBr] [13,14]. In the absence of a stretching force, $\delta\Delta G(\mathrm{EtBr})$ is given by

$$\delta\Delta G^0(\mathrm{EtBr}) = \Delta H_{\mathrm{EtBr}}\Big(T_0 - T_m(\mathrm{EtBr})\Big)\Big/T_m(\mathrm{EtBr}) \quad (2)$$

where T_0 is the melting temperature of dsDNA in the absence of drug, $T_m(\mathrm{EtBr})$ is the melting temperature of the EtBr-DNA complex, and ΔH_{EtBr} is the DNA melting enthalpy in the presence of the drug. Here we use $\Delta H_{\mathrm{EtBr}} = 8$ kcal/mol/bp [13]. In contrast to bulk binding studies, in our experiments ethidium is always in excess over DNA. To compare our measured $\Delta G([\mathrm{EtBr}])$, [Eq. (1)] to the free energy calculated from the previously measured T_m [14] according to Eq. (2), we calculated the concentration of free ethidium in these experiments from the added drug concentration D and DNA phosphate con-

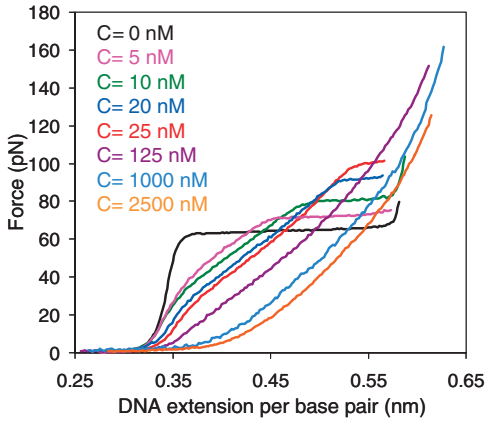

FIG. 2 (color). DNA stretching curves in the absence (black curve) and presence of different EtBr concentrations (5 nM, pink; 10 nM, green; 20 nM, dark blue; 25 nM, red; 125 nM, purple; 1000 nM, light blue; 2500 nM, orange).

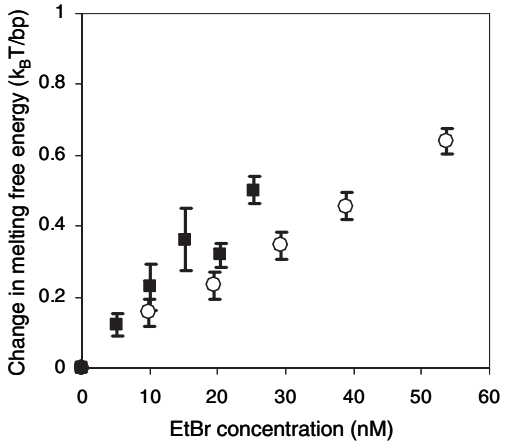

FIG. 3. Increase in melting free energy from single molecule stretching experiments according to Eq. (1) (■) and from thermal melting experiments according to Eq. (2) (○).

centration P according to the expression $[\mathrm{EtBr}] = D/(1 + KP)$, where $K = 10^6\ \mathrm{M}^{-1}$ [15] is the binding constant of EtBr per base to dsDNA. The data presented in Fig. 3 corresponds to low binding, where the latter expression is valid. The agreement between these two measurements supports the melting nature of the observed transition. Another argument supporting the interpretation of the transition as force-induce melting is the hysteresis, which is observed only when the melting transition is observed (Fig. 1).

An alternative explanation for the cooperative force-induced structural transition in the absence of ethidium assumes the existence at higher forces of a novel form of double-stranded DNA, ''S-DNA,'' which is almost twice as long as B-DNA [16–19]. While our data on the transition in the presence of EtBr cannot rule out the existence of S-DNA, its existence would require that two elongated dsDNA forms simultaneously occur, of which one binds ethidium, while the other does not. Also, ethidium-induced stabilization of dsDNA would have to be the same relative to both S-DNA and melted DNA. Finally, since hysteresis is a strong signature of strand separation, the ethidium-DNA to S-DNA transition would have to occur concurrently with strand separation.

To characterize EtBr-dsDNA binding, we follow the changes in the DNA stretching curve upon addition of EtBr from the pure dsDNA to the saturated EtBr-dsDNA stretching curve, as shown in Fig. 2. However, changes in the stretching behavior of DNA produced by ethidium are not the same at low and high forces. At low EtBr concentrations, EtBr-dsDNA elongation is minor at $F \leq 10$ pN, but increases strongly at higher forces (Fig. 1), suggesting that these forces may allow further drug binding at fixed EtBr concentration. To quantify this behavior, we measured the increase in DNA length at a fixed force as a function of EtBr concentration. We then calculated the fractional occupancy of binding sites Θ at each drug concentration by dividing the change in DNA length by its maximum value at saturated drug binding. This procedure was performed at a number of forces ranging from ~5 to ~60 pN. An example of an EtBr titration curve obtained from such DNA lengths measured at 30 pN is shown in Fig. 4. A quantitative fit to the McGhee and von Hippel binding isotherm [20,21] yields as best fit values at low force $F \leq 10$ pN, $K = 10^7\ \mathrm{M}^{-1}$ and $n = 2$, while at high forces, $F \geq 20$ pN, the best fit values are $K = 1.5 \times 10^7\ \mathrm{M}^{-1}$ and $n = 1$. Thus, at low forces the binding site size and binding constant are in reasonable agreement with previous bulk studies which report values for the EtBr binding constant in the range of $10^6\ \mathrm{M}^{-1}$ [15] and $10^8\ \mathrm{M}^{-1}$ [22] obtained under solution conditions ranging from 20 to 150 mM salt concentration. At high forces, the binding constant is slightly higher, and the maximum binding corresponds to one ethidium intercalated at every base pair stack. This effect was predicted in the work of Yan and Marko [23], and is supported by the 0.68 nm/bp contour length of the saturated ethidium-dsDNA, which is twice the length of B-DNA. This is in contrast to the 1.5-fold elongation, expected for ethidium intercalation of only every other base pair, observed previously in scanning force microscopy [24] and hydrodynamic [25] studies. The saturated intercalation of ethidium at every other stack was shown to be associated with the fine conformational balance of two DNA backbones, rather than direct steric clashes between the intercalated ethidium molecules [26,27]. Therefore the strong axial stress, which imposes its own backbone conformation, is likely to release this site exclusion requirement and promote further ethidium intercalation, as observed in this study at forces greater than ~10 pN.

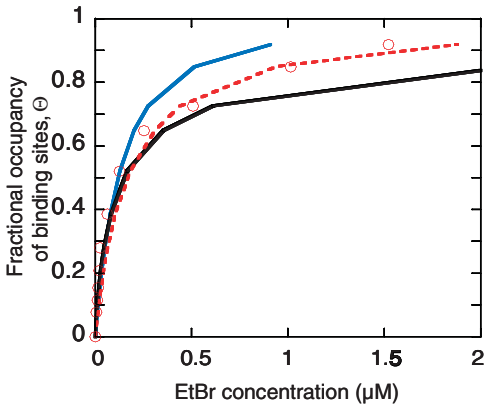

FIG. 4 (color). Fractional binding of ethidium as a function of concentration, determined from the fractional increase on DNA contour length at 30 pN. Symbols are measured data, while the lines are fits to the data with $K = 10^7\ \mathrm{M}^{-1}$ and $n = 0.8$ (blue), $n = 1.0$ (red), and $n = 2.0$ (black). Errors are similar to the data point size.

Because force promotes EtBr binding at every base pair, DNA saturation with EtBr is accompanied by the doubling of the dsDNA contour length. This leads to force-extension curves of ethidium-dsDNA and melted DNA that intersect at a high force. Further ethidium-dsDNA stretching beyond the extension at intersection stabilizes the dsDNA relative to its melted state. Thus, at every EtBr concentration there is only limited mechanical work, $W([\mathrm{EtBr}])$, that can be done on the molecule in order to melt it. This work decreases as ethidium binding increases. At the same time, the duplex stability in the absence of force, $\Delta G(\mathrm{EtBr})$, grows with increasing [EtBr]. Therefore, at [EtBr] higher than a critical value $[\mathrm{EtBr}]_{\mathrm{cr}} = 25$ nM (i.e., fractional DNA saturation with EtBr of $\Theta \sim 0.2$), defined by the condition $W([\mathrm{EtBr}]) = \Delta G([\mathrm{EtBr}])$, the force becomes incapable of melting ethidium-dsDNA at any extension. At $[\mathrm{EtBr}]_{\mathrm{cr}}$, the melting force plateau vanishes, and the DNA molecule can exist either as an ethidium-dsDNA or ssDNA at the same force and extension. At this critical point the whole DNA molecule should transform from ethidium-dsDNA to ssDNA. However, these two thermodynamically equivalent DNA states are separated by a large energetic barrier, related to the significant boundary

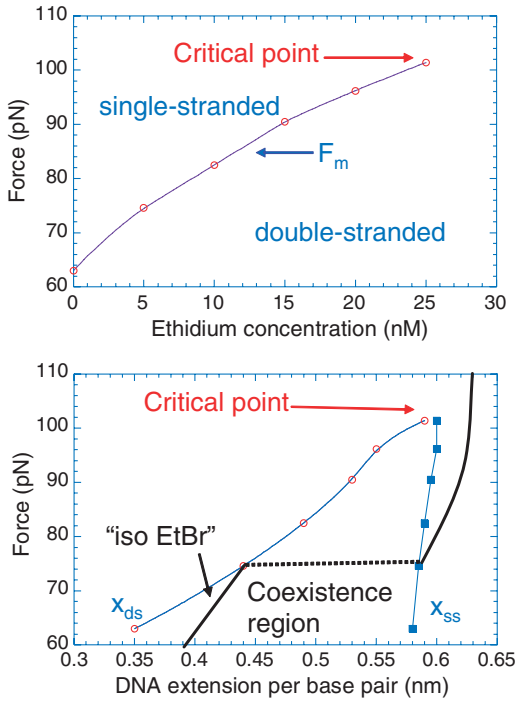

FIG. 5 (color). Phase diagram for a single DNA molecule in the (a) Force-[EtBr] plane and (b) Force-extension plane.

free energy between the helical and melted forms of DNA. Therefore, the formation of the nuclei of the new phase within the old one is slow. This slow nucleation of the DNA melting and annealing becomes especially important at solution conditions close to the critical point. Therefore, EtBr-dsDNA and ssDNA often exist as metastable states near the critical point, resulting in a large variation of the apparent transition force and width, as well as larger hysteresis. The situation is analogous to the rapid heating and cooling of the bulk liquid-gas system near its critical point [28], which leads to the appearance of the metastable phases.

Based on the analogy between a liquid-gas equilibrium and our EtBr-dsDNA–ssDNA transition, one can think of the force, extension, and EtBr concentration in our experiment as analogs of the pressure, volume, and temperature, respectively. The only qualitative difference is that while the volume of liquids and gases decrease with pressure, the DNA extension grows with force. In a liquid-gas transition, increasing temperature results in smaller differences in liquid and gas volume, leading to a higher transition pressure. Similarly, increasing [EtBr] results in a smaller difference in the extensions of dsDNA-ethidium and ssDNA, leading to a higher melting force. In the F-[EtBr] plane the phase coexistence curve for dsDNA and ssDNA is a line that ends at $[\mathrm{EtBr}]_{\mathrm{cr}}$, while the F-x plane maps out the coexistence region, as shown in Fig. 5. Thus, these studies allow us to map the phase diagram of a single DNA molecule and quantitatively characterize the thermodynamics of this important class of DNA interactions.

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