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DNA Intercalation

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Dynamics and Multiple Stable Binding Modes of DNA Intercalators Revealed by Single-Molecule Force Spectroscopy**

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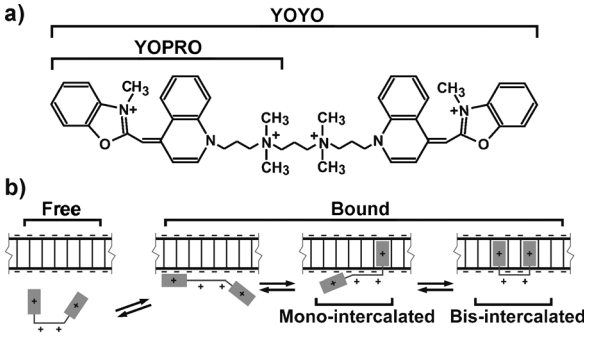
DNA intercalators are widely used to fluorescently label DNA,[1] and as drugs in cancer therapy.[2] Intercalation occurs through the insertion of a planar polycyclic aromatic ring between adjacent bases. Increased affinity is achieved by using a pair of polycyclic moieties and by electrostatic interaction between the positively charged intercalator and the negatively charged DNA. Ongoing research shows a trend toward tighter binding affinities and specificity for DNA-intercalating drugs.[3] Further refinement of intercalators would be accelerated by a better understanding of their binding modes, intercalation dynamics, and sensitivity to ionic conditions.

The mechanical properties of DNA are altered by intercalation. For example, the contour length (L) of DNA increases with intercalation.[4] The overstretching transition of DNA, where the extension of the DNA increases by 70% at 65 pN, is also altered.[5] Thus, single-molecule force spectroscopy (SMFS) is a sensitive probe to DNA intercalation.[5b]

SMFS has been used to probe the binding of both dye molecules (ethidium bromide,[4–6] YOYO-1,[6,7] YO-PRO-1,[6,7b] etc.) and drugs (psoralen,[5a] daunomycin,[6] etc.). YOYO-1[8] is a bisintercalating cyanine dye (Scheme 1)that is often used to image individual DNA molecules.[9] YOYO-1 (YOYO),[7b,8] in conjunction with its monomer YO-PRO-1, (YOPRO) provides a useful basis for studying DNA intercalation.[10]

Stretching the DNA enhances the intercalation of YOYO,[6b,7a,b] similar to experiments with ethidium bromide.[5b] Kinetic schemes of force-enhanced intercalation[5b,7b,11] have assumed that the bisintercalated state comes into rapid (ca. 2 s) equilibrium with the unbound state or free dye. For instance, Murade et al.[7b] stretched a 16 μm long DNA strand in the presence of YOYO (100 nM) at a rate of 1 μm s^{-1}, and observed an increase in both extension and fluorescence. Immediately after a fast (3 μm s^{-1}) change in the extension, the force (F) decayed with a time constant of 0.2–2 s, depending on the peak force. These results were interpreted within a model in which the affinity of YOYO for DNA increased with F, and more molecules were "bound" as a new equilibrium was reached. Experiments with YOPRO also showed an increase in fluorescence but no decay in F after a force jump; the interpretation was that the binding of YOPRO from solution was too fast to be resolved.



Scheme 1. a) Structures of YOYO and YOPRO. b) Binding states of YOYO.

The first step in bisintercalation is a bound, but unintercalated state that is expected to be transiently populated on the way to the more stable bisintercalated state (Scheme 1b). We tested this assumption by combining SMFS with a simple buffer exchange to remove the free dye. This protocol distinguishes between the effects of intercalation of bound dye that is not fully intercalated and intercalation of unbound dye from solution. We show that force-enhanced intercalation at a NaCl concentration of 150 mM can occur from a reservoir of dye that is not fully intercalated, yet remained out of equilibrium with free dye for periods that are long compared to many single-molecule experiments (>5 min for YOPRO, and >2 h for YOYO). Moreover, we show that the rate of force-enhanced intercalation of YOYO depends strongly on the concentration of the monovalent salt. In contrast, adding divalent cations to mimic physiological ionic strength (150 mM NaCl + 1 mM $MgCl_2$) did not alter the dynamics.

Single DNA molecules were stretched by using a standard surface-coupled optical-trapping assay (Figure 1a;[12] see the Supporting Information). We used 2 μm long DNA stained at two different dye concentrations (10 and 100 nM) of YOYO or YOPRO and a range of loading rates (0.5, 5, and 50 μm s^{-1}) at buffer conditions similar to prior work (10 mM TrisCl (pH 7.5), 150 mM NaCl, 1 mM EDTA).

As an initial probe for force-enhanced intercalation dynamics, we applied a force jump followed by a force quench. This experiment also illustrates our measurement process (Figure 1b, steps 1–6): 1) A 2D elasticity-centering routine was performed at minimal F (ca. 8 pN) to align the anchor point of the DNA strand to the optical axis of the trap

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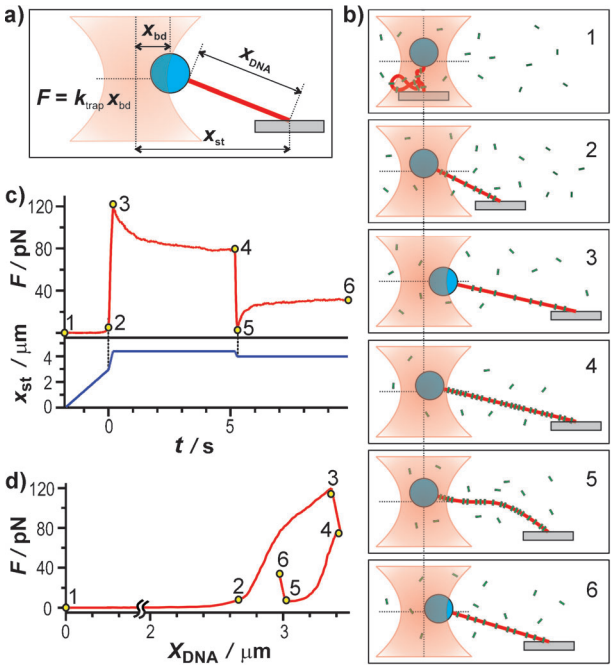

Figure 1. Experimental setup. a) Trapping geometry showing a surface-anchored DNA molecule (red) stretched by a bead held in an optical trap. Force along the stretching axis was determined by using the bead position (x_{bd}), stage position (x_{st}), and trap stiffness (k_{trap}).[12] b) Schematic representation of the force jump/force quench protocol illustrating force-induced binding of intercalators at different stages in the process (1–6). c) Force curve of stretching DNA in the presence of YOYO (100 nM) by rapid changes in x_{st}. d) Force–extension curve of the same data as shown in (c). Data smoothed to 600 Hz.

and to measure the initial contour length (L) of the strand. 2) The DNA strand was next stretched to L by applying approximately 10 pN. 3) A continuous data acquisition at 120 kHz was started and the stage was rapidly moved at a constant velocity to a preset distance. 4) The trap/anchor point separation was fixed for a specified duration. 5) F was reduced by reverse motion of the stage to a new position. 6) The resulting force recovery was probed. This protocol and similar ones result in a distinct "mechanical fingerprint"[5a] for studying intercalation of compounds into and out of DNA with high spatiotemporal resolution.

In the presence of YOYO (100 nM), this mechanical fingerprint shows a force decay after the force jump and a force recovery after the force quench (Figure 1 b–d), which is consistent with prior results.[6b,7a,b] Within the force-enhanced binding model (Figure 1), tension in the DNA increased the affinity for intercalated YOYO; as a result, additional YOYO from solution intercalated into the DNA, lengthening the DNA strand and thereby reducing F. The subsequent force quench led to deintercalation and release of YOYO from the DNA and, thereby, to a force recovery. The experimental signature for equilibration with the free dye was taken to be when F became constant.

To confirm the results of our assay and highlight the known changes in the elasticity of DNA that results from intercalation, we compared the initial phase of the force–extension curve of unstained DNA (gray) to data taken in the presence of YOYO and YOPRO at different concentrations (Figure 2). For YOYO at a concentration of 100 nM (red), these results are similar to prior work[7b] and show that the rise

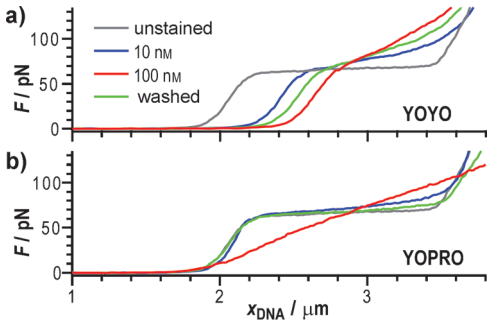

Figure 2. Force–extension curves (FEC) at $\nu_{st} = 5\ \mu m\,s^{-1}$ of an unstained DNA (gray), a DNA–dye complex in 10 nM (blue) and 100 nM dye (red), and after washing out the 100 nM dye solution with a buffer containing no dye molecules (green). The force traces correspond to experimental steps 1–3 depicted in Figure 1 b.

in the force–extension curve (FEC) was shifted toward longer extension and a slanting of the FEC above 65 pN. We also probed the elasticity of DNA at a YOYO concentration of 10 nM (blue). Even at this low concentration, we measured a significant rightward shift, which was larger than in prior work, in the FEC. As a consequence, L changes during stretching, even at low F (see Figure S1 in the Supporting Information); indeed, recent work suggests that this effect has led to the incorrect conclusion that the persistence length changes with YOYO staining.[7c] Based on this work, we assumed that p was unchanged. Thus, we measured L immediately prior to the rapid force jump and used this L to estimate the number of bound YOYO molecules. This analysis yields 0.11 dye molecules per base pair (bp) at a dye concentration of 10 nM and 0.21 dye molecules per bp at a dye concentration of 100 nM.[7c] Or, as will be highlighted below, this analysis reports the degree of intercalation per bp.

In the presence of 10 nM YOPRO, the intercalation effects are less pronounced, as expected for the monomeric form of YOYO. At a YOPRO concentration of 100 nM, our observations qualitatively agree with prior work[7b] and show a linear increase in the FEC beyond 2 µm, which has been attributed to a fast exchange between bound and unbound YOPRO as the affinity of YOPRO for DNA increases with F.

This difference in the equilibration rates of YOYO and YOPRO was highlighted by removing the free dye by flowing more than 20 sample chamber volumes of dye-free buffer through the flow cell and leaving the DNA in this buffer for more than 5 min (see the Supporting Information). The FEC for YOPRO (green) was almost the same as for the unstained DNA (gray) after washing. In contrast, the FEC of DNA that was previously stained with YOYO was situated between the 10 and 100 nM curves and distinctly different from that of unstained DNA. This suggests that a large fraction of YOYO remained intercalated in an unequilibrated state for an extended period of time. This slow off rate for YOYO is well known and has been exploited to visualize a single DNA

molecule in the presence of unstained DNA.[13] However, an unstated assumption was that this long-lived state had both polycyclic moieties intercalated.

To explore intercalation kinetics, we focused on the force decay after a force jump. Figure 3 shows force curves that reached a similar peak force during a force jump as a function of pulling rates in 100 nM dye. By recording high time resolution data during the force jump along with the resulting force decay (Figure 1b, steps 2–4), we gained a more complete picture of force-enhanced intercalation.

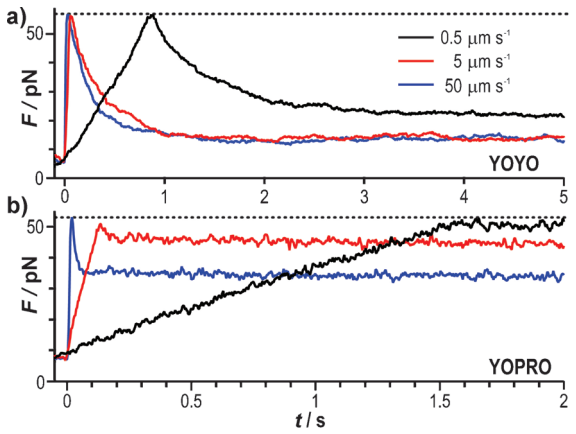

Figure 3. Force as a function of time at various pulling velocities. The traces correspond to experimental steps 2–4 depicted in Figure 1.

For YOYO, these results suggest that, at the fastest pulling rate, no extra intercalation took place during the time of the force jump ($t_{jump} = \Delta x_{st}/v_{st} = 6$ ms, where $\Delta x_{st} \approx 0.3$ μm) and, hence, our experiment fully resolved the decay dynamics of the force-enhanced intercalation. As evidence, the pulling curves at rates of 5 and 50 μm s^{-1} are nominally superimposable and show the same decay amplitude, indicating the same degree of extra intercalation after the force jump. This similarity is in contrast to the pulling curve at a speed of 0.5 μm s^{-1} (black), which shows a significantly reduced decay amplitude in agreement with intercalation occurring during the slower force jump.[6b]

Our results with 2 μm long DNA are different than prior results with 16 μm long DNA, which showed changes in FEC at pulling speeds up to 20 μm s^{-1}.[7b] We attribute this difference to our use of shorter DNA, *F* below the overstretching transition, and higher stretching rates (50 μm s^{-1}). These high rates allowed us to clearly resolve the full dynamics of the force-enhanced intercalation.

For YOPRO, the combination of short DNA and fast stretching allowed us to directly observe the previously unresolved dynamics of force-enhanced intercalation. At our slowest pulling rate (0.5 μm s^{-1}), there was no force decay. This data is consistent with prior work, which concluded that force-enhanced YOPRO intercalation is in equilibrium during stretching of 16 μm long DNA at 3 μm s^{-1}.[7b] At the intermediate pulling speed (5 μm s^{-1}), a small decay was discernible. A significant decay amplitude (18 pN) was observed only at the highest stretching rate. The corresponding decay time (13 ms) was two times slower than the stretching time (6 ms at $v_{st} = 50$ μm s^{-1}). Thus, we expect that only a modest amount of intercalation took place during the force jump and the resulting force decay accounted for the majority of the force-enhanced intercalation. Our results therefore provide a time scale for YOPRO intercalation.

As mentioned above, prior work has discussed force-enhanced intercalation in the context of an equilibration with free dye rather than intercalation from a bound state that was not fully intercalated.[7b] We tested this assumption by removing the unbound dye and then performing a force jump, force quench, force jump protocol (Figure 4). With this

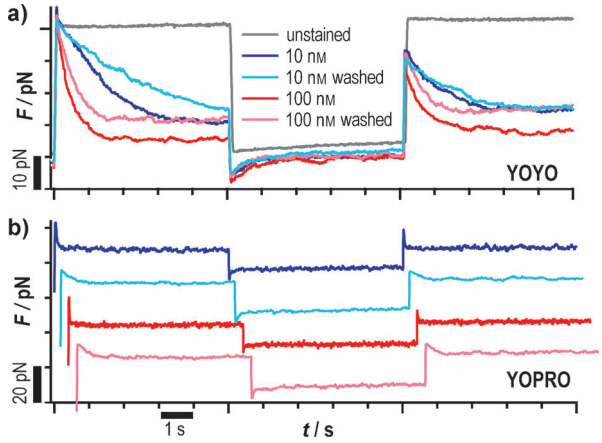

Figure 4. Force curves of DNA–dye complexes in the presence and absence of free dye (e.g., 10 nM and 10 nM wash, respectively), where the free dye was removed by buffer exchange (wash) after staining. a) Force curves for YOYO-stained DNA with $v_{st} = 5$ μm s^{-1}. b) Force curves for YOPRO-stained DNA with $v_{st} = 50$ μm s^{-1} (same color scheme as in (a)). Traces in (b) are displaced for clarity.

experimental protocol, the hypothesis that force decays arise from binding and intercalation of free dye from solution would predict neither a force decay after a force jump nor a force recovery after a force quench, since there is no free dye.

In contrast to this expectation, our results show both a force decay and a force recovery in the absence of free dye. In Figure 4, we show results for the same individual DNA molecule stretched in the presence of dye (10 or 100 nM) and after washing. As before, the free dye was removed by flowing more than 20 sample chamber volumes of dye-free buffer through the flow cell and then waiting for more than 5 min. For YOYO at a concentration of 100 nM, the results in the presence and absence of dye are remarkably similar, showing the same initial decay rate, and only a 10% smaller decay amplitude in the absence of free YOYO. For YOYO at a concentration of 10 nM, the decay amplitudes were the same, while the decay rate after washing was about two times slower. Taken together, these results argue that the primary mechanism for force decay in the presence of YOYO is force-enhanced intercalation from a reservoir of strongly bound YOYO in which both polycyclic moieties are not already intercalated. The subsequent dynamics after a force quench and the second force jump, all in the absence of the unbound

dye, further argue for a dynamic interchange of polycyclic moieties into and out of the DNA with only a modest reduction of bound YOYO, as shown by the reduced peak force during the second force jump.

One simple explanation for these dynamics in the absence of dye would be for one of the polycyclic moieties of the dimeric YOYO to be intercalated while the other one was not. Within this hypothesis, YOPRO should not show similar dynamics. Yet, when the same stretching protocol was applied to DNA that was initially stained with YOPRO and then washed, we observed a force decay after a force jump and a force rise after a force quench similar to YOYO, albeit with a 43% reduced decay amplitude and a seven-fold increased decay time in comparison to stretching in 10 nM YOPRO. Thus, the origin of tight binding in the absence of intercalation is more complex.

An alternative hypothesis for the tight binding in the absence of intercalation is electrostatic attraction between the negatively charged DNA and the positively charged dye molecule (Scheme 1). Hence, we probed the effect of monovalent ions on the strongly bound state by performing force jump experiments on DNA subjected to a series of low salt (LS) and high salt (HS) rinses (Figure 5a). Prior to the salt washes, the DNA was stained with YOYO (10 nM) with no added NaCl. The force jump in the staining solution showed minimal force decay, consistent with prior work at a NaCl concentration of 10 mM.[7c] We removed the free dye as before using a dye-free LS buffer (1 mM NaCl). Force jumps in the dye-free LS buffer also showed minimal decay. Yet, after an additional buffer exchange to a dye-free HS buffer (500 mM NaCl), the force jump led to the signature force decay. Interestingly, this decay rate was about five times faster than a corresponding dye-free decay curve at a NaCl concentration of 150 mM (Figure 4a, cyan). This salt-induced change in kinetics was reversible; decay was suppressed after exposing the DNA to an LS buffer and then returned after re-exposure to HS conditions, albeit with a slightly longer decay time and decreased amplitude. These experiments took approximately 2 h because of multiple buffer exchanges and multiple molecules measured at each ionic condition. Thus, both force-enhanced intercalation and salt-induced kinetic modulation can occur with YOYO that is strongly bound to DNA for periods that are long relative to most single-molecule experiments.

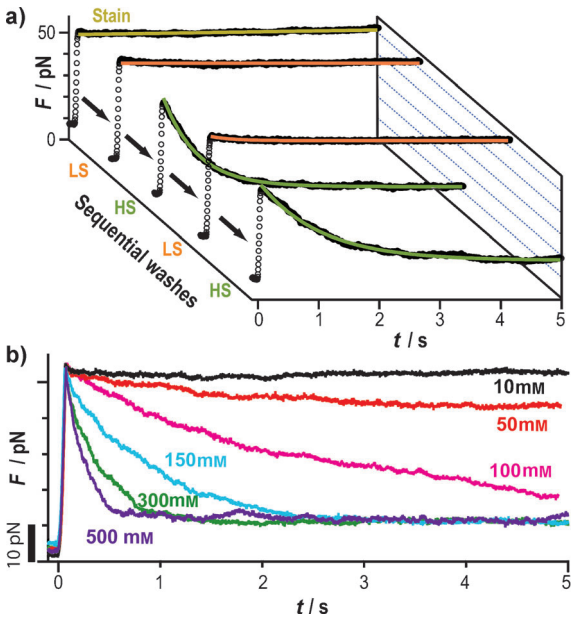

Figure 5. Salt dependence of force decay. a) Force decay in staining solution (gold: 10 nM YOYO, no added salt) followed by serial buffer exchanges into dye-free low salt (LS, orange) and high salt (HS, green) conditions, with each ionic condition lasting approximately 25 min. b) The force decay with no free dye at 5–25 min after exchanging into different ionic conditions (10–500 mM NaCl). Each ionic condition was probed after staining the DNA (100 nM YOYO, no added NaCl) for approximately 20 min. For clarity, traces were displaced vertically to align the peak in the curves.

One mechanism for modulating decay kinetics would be for bound YOYO to be bisintercalated at LS concentrations, but have only one or no polycyclic moieties intercalated at HS concentrations. Determination of the contour length immediately prior to the force jump allowed us to assess the degree of intercalation (Figure S2). During staining and at the first LS condition, L was approximately 650 nm longer than that of unstained DNA. After the first buffer exchange into HS conditions, the final three curves of Figure 5a showed an increase in L of approximately 50 nm in comparison to unstained DNA, thus implying a similar degree of intercalation in these three results. These data suggest that the primary cause of salt-induced modulation of kinetics does not solely arise from an alteration in the degree of intercalation at different ionic conditions.

To quantify the effect of monovalent ions on force-enhanced intercalation kinetics, we performed a similar set of experiments, using force jumps over a range of ionic strengths (10 mM to 500 mM NaCl). Again, we probed intercalation kinetics by flushing out the unbound dye. Also, given the slow decay rate at 1 mM NaCl (Figure 5a), we increased the concentration of YOYO to 100 nM to increase the rate of decay after a force jump (Figure 4a). The resulting curves clearly show that the decay rate increased with increasing NaCl concentration (Figure 5b). In contrast, no significant decay was seen in an individual unstained DNA strand stretched under all six ionic conditions (Figure S3). Hence, monovalent ions significantly accelerated the rate of YOYO intercalation, at least for force-enhanced intercalation.

We speculated that divalent cations present at nominal physiological salt concentrations (150 mM NaCl, 1 mM $MgCl_2$) might reduce the electrostatic attraction and thereby alter the binding mode of these and similar intercalators that are used as drugs. However, at these salt concentrations, the force-dependent intercalation dynamics were unchanged (Figure S4). Thus we would expect similar binding modes for in vivo applications.

In summary, we isolated the effects of binding and intercalation of two model DNA intercalators (YOYO and YOPRO) by combining SMFS with simple buffer exchange. We showed that force-enhanced intercalation can occur from a reservoir of bound dye that was not fully intercalated, yet remained out of equilibrium with free dye for periods that are long compared to many single-molecule experiments

(>5 min for YOPRO, and >2 h for YOYO). Moreover, for YOYO, this reservoir of polycyclic moieties that are not bisintercalated accounts for most of the decay observed in the presence of free dye. Our work highlights that binding/unbinding and intercalation/deintercalation are distinct processes that can occur on very different time scales. More succinctly, "not intercalated" is not synonymous with "not bound". We expect that these insights and experimental protocols will enable the refinement of binding and intercalation of fluorescent dyes and drugs.

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