Supplementary Data

For the manuscript “Force spectroscopy reveals the DNA structural dynamics that govern the slow binding of Actinomycin D”, by Thayaparan Paramanathan, Ioana Vladescu, Micah J. McCauley, Ioulia Rouzina, and Mark C. Williams

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Supplementary Figure S1: Progressive stretching of DNA into the melting plateau in the presence of 50 nM ActD indicates that only the fraction of dsDNA stretched into the plateau binds ActD. Stretch and release cycles are represented by solid and broken lines. During the 1st cycle (red) the DNA was stretched only to the extension at which $F<F_m$, such that the DNA was not melted by force. No measurable ActD binding occurred during this cycle, as the curves remained unchanged by ActD (overlaps the black DNA stretching curve obtained in the absence of ActD). In the 2nd (orange) and 3rd (green) cycles the DNA was stretched to an extension within the melting transition. The apparent length of the release curve corresponds to the fraction melted and subsequently bound by ActD, indicating that binding to melted DNA was near saturation. Finally, in the 4th (blue) and 5th (purple) stretches the DNA was completely force-melted by stretching to $F>F_m$, and both release curves overlap indicating that no additional ActD bound during the 5th stretch compared to the 4th stretch.
Supplementary Figure S2: Quantitative analysis of ActD-2DNA binding at $F>F_m$.

(a) DNA stretching (dark orange solid) and release (dark orange open circles) curves in the presence of 6 nM ActD used to illustrate the method of quantitative analysis that characterize the ActD-2DNA binding at $F>F_m$. Fractional binding $\Theta$ is determined assuming that at $F>F_m$ the DNA is a linear combination of ligand-free single ssDNA strand with weight $(1-\Theta)$ and ActD-2DNA with weight $\Theta$. Upon DNA release, the ligand-free ssDNA fraction re-anneals rapidly at $F\sim F_m$, while the fraction of DNA within the ActD-2DNA complex, $\Theta$, does not have time to dissociate during DNA release, becoming locked at $F<F_m$. Thus, at $F<F_m$, the DNA release curve is a linear combination of bare dsDNA elasticity with the weight $(1-\Theta)$ and of the elasticity of ActD-2DNA with weight $\Theta$. To obtain the corresponding high force dissociation constant $K_d\ (F>F_m)$, four complementary approaches were used. Two of them used equilibrium $F(x)$ curves at $F>F_m$, while the other two used the non-equilibrium DNA release curves at $F<F_m$. At $F>F_m$ we followed $\Delta F$ at $x=0.566\ \text{nm}$ (as green arrow indicates) and $\Delta x$ at $F=76\text{pN}$ (as the red arrow indicates) to estimate $\Theta([\text{ActD}])$. At $F<F_m$ we have followed the extension during DNA release at $F=20\text{pN}$ (as violet arrow indicates) and $F=34\text{pN}$ (as blue arrow indicates).

(b) The fraction of DNA within the ActD-2DNA complex, $\Theta$, at high forces ($F>F_m$) as a function of ActD concentration. $\Theta([\text{ActD}])$ (open circles) was estimated using four
complementary approaches discussed above: at $x=0.566\text{nm}$ (green), at $F=76\text{pN}$ (red), at $F=20\text{pN}$ (violet) or $F=34\text{pN}$ (blue). All four approaches yield a similar $\Theta([\text{ActD}])$ dependence, thereby supporting our model. The average $\Theta([\text{ActD}])$ shown with the brown solid circles is fitted to the simple binding isotherm $\Theta(C) = \frac{C / K_d}{1 + C / K_d}$ (brown solid curve, the broken curves represent the error margins), to obtain the high force dissociation constant $K_d(F>F_m) = 18\pm6\text{nM}$. This value is in good agreement with the $K_d$ estimated at $F\geq65\text{pN}$ from the $K_d(F)$ measurements (Figure 4b).
Supplementary Figure S3: Kinetics of ActD-2DNA binding at $F>F_m$ estimated from pulling rate ($\nu$) dependence of ActD-DNA stretching curves. DNA stretching (solid) and release (broken) curves in absence of ActD (black) and in the presence of 50 nM ActD at $\nu = 20$ nm/s (violet), 50 nm/s (blue), 100 nm/s (green), 500 nm/s (orange) and 1000 nm/s (red). The fastest pulling rate curve (red) is the closest to those observed in the absence of ActD (black), suggesting that very few ActD molecules had time to bind during the ~10 s of DNA stretching. Slower pulling leads to a shorter ActD-2DNA complex as additional ActD binds at $F>F_m$, until saturation is reached at $\nu \leq 100$ nm/s. This result suggests that at $F>F_m$ the on rate for ActD is less than the DNA stretching time ($\tau_{str} \sim 100$ s) at 100 nm/s, but longer than the stretching time ($\tau_{str} \sim 20$ s) at 500 nm/s, such that $20 \text{ s} \leq \tau_{on}(F>F_m) \leq 100$ s. Therefore the ActD-2DNA binding rate at $F>F_m$ lies within the range $0.01 \text{ s}^{-1} < k_{on}(F>F_m) < 0.05 \text{ s}^{-1}$. Assuming the bimolecular relationship $k_{on} = [\text{ActD}]k_a$ (verified in Supplementary Figure S6), we estimate the corresponding high force bimolecular association rate constant, $k_a(F>F_m)$, to be within the range $2 \times 10^5 \text{ M}^{-1}\text{s}^{-1} < k_a(F>F_m) < 10^6 \text{ M}^{-1}\text{s}^{-1}$. The lower bound of this estimate coincides with the $k_a(F\sim 65\text{pN}) \sim 10^5 \text{ M}^{-1}\text{s}^{-1}$ value obtained as a high-force limit of the force dependence measurement of $k_a(F)$ at $F<F_m$ (Figure 5d). Taken together, these high-force $k_a$ and $K_d$ values allow for the estimate of ActD’s off rate $k_{off}(F>F_m)= K_d \times k_a = 18 \text{ nM} \times (2 \times 10^5 - 10^6) \text{ M}^{-1}\text{s}^{-1} = 0.004 - 0.02 \text{ s}^{-1}$. The lower end of this estimate agrees well with the average
$k_{\text{off}}(F-65\text{pN})\sim0.004 \text{ s}^{-1}$ value obtained from the force dependence of $k_{\text{off}}$ measured at $F<F_m$ (Figure 5c).
Supplementary Figure S4: Elastic properties of ActD-2DNA compared with classical intercalators support the intercalation of ActD. Stretching curves of the saturated DNA complex (open circles) with ActD (ActD-2DNA) (red) compared with ethidium (violet), and the two ruthenium (Ru) ligands (blue and green). ActD-2DNA stretching curve is observed at [ActD] ≥ 4 µM as a converged DNA stretching and release curve, that lacks any signature of a melting transition and appears much longer than B-form DNA. This implies that ActD-2DNA consists of two DNA strands bound together due to their association with intercalating ActD. The red line approximating the red open circles represents the fit of ActD-2DNA stretching curve to the worm-like chain (WLC) model of polymer elasticity:

\[ x(F) = x_{max} \left(1 - \frac{1}{\sqrt{4F \cdot A / k_BT}} + \frac{F}{S}\right), \]

with the persistence length \( A = 10 \pm 3 \) nm, contour length, \( x_{max} = 0.43 \pm 0.01 \) nm/bp, and elastic modulus \( S = 320 \pm 20 \) pN. These parameters are within 20% of the analogous parameters for the saturated complex of DNA with the three other more conventional intercalators shown here (35). Thus, saturated ActD binding leads to ~25% increase in contour length, 5-fold decrease in persistence length and ~3-fold reduction of the elastic modulus compared to B-DNA. At the same time the ActD-2DNA complex remains a rather rigid polymer with persistence length 16-fold larger than that of the flexible ssDNA. The
longer contour length of the ActD-2DNA complex at low forces can be interpreted as zero-force ActD intercalation at every \( n_0 \) base pair(35), where \( n_0 = \frac{x_{ds}}{x_{max}-x_{ds}} \). The relatively large binding site size of ActD (\( n_0 \sim 4 \)), most likely reflects the high sequence specificity of ActD intercalation, which requires at least one G residue on its border. Another possible reason for the large observed binding site size may be shared by ActD with other intercalators (35-37), and involves neighbor exclusion due to excessive ligand-induced DNA deformation. This limitation on the amount of intercalated ActD is relieved by higher force, leading to additional intercalation at almost every base pair and to a doubling of the dsDNA contour length, as reflected by the relatively low elastic modulus of ActD-2DNA. While ActD-, ethidium- and Ru complex- saturated DNA stretching curves all look rather similar to each other, there is a major difference between these ligands. The ethidium- and Ru- saturated DNA complexes have rapid on/off kinetics (less than millisecond on/off times) and are in equilibrium on the time scale of our stretching experiments \(~100 \) s. At the same time, in the absence of force it takes \(~1000-10000 \) s for the ActD-2DNA on and off processes to equilibrate, even at \([\text{ActD}] \gtrsim K_d(0) \sim 0.6 \mu\text{M} \). Thus, despite the fact that the zero-force ActD-2DNA binding affinity is comparable to that of ethidium and previously studied Ru complexes \((K_d(0) \sim 1-2 \mu\text{M})\), the much slower ActD on and off kinetics lead to incomplete association and dissociation during our DNA stretching cycle. Therefore, the ActD-2DNA stretching curves are out of equilibrium.
Supplementary Figure S5: ActD-2DNA equilibrium and kinetic binding parameters measured in the absence of flow in solution with 50 nM ActD are consistent with parameters obtained in the presence of flow with 500 nM ActD. (a) Extensions (open circles) were obtained at different constant forces 20 pN (violet), 30 pN (blue), 40 pN (green) and 50 pN (red) after adding a solution containing 50nM ActD to fill the flow cell and fitted to Eq. 1 (solid lines). (b) Force dependence of on and off rates (solid green and red lines) according to Eq.7 yield $k_{on}(0) = (3.8 \pm 0.6) \times 10^{-5}$ s$^{-1}$, $k_{off}(0) = (3.1 \pm 0.6) \times 10^{-4}$ s$^{-1}$, $x_{on} = 0.26 \pm 0.05$ nm and $x_{off} = 0.07 \pm 0.02$ nm. The $k_{on}(0)$ obtained at 50 nM from above fit is 10 fold smaller than the one obtained at 500 nM from Figure 5c which is an additional confirmation of bimolecular nature binding. In addition $\Delta x_{eq} = x_{on} - x_{off} = 0.19 \pm 0.04$ nm is also in agreement within error with the equilibrium extension upon binding to DNA, $\Delta x_{eq} = 0.20 \pm 0.05$ nm obtained from the force dependence of $K_d$ (Figure 5b). Also, the on and off rates in the presence of 50 nM ActD become equal at $F \sim 45$ pN (see $k_{on}(F)$ and $k_{off}(F)$ line crossover in panel b), showing that $K_d \sim 50$ nM is achieved at this stretching force. This result is in agreement with the directly measured $K_d(45$ pN)$ \sim 50$ nM shown in Figure 5b.
**Supplementary Figure S6: ActD-DNA binding is bi-molecular.** The concentration dependence of the ActD-2DNA complex on (green points) and off (red points) rates at \(F=30\) pN were obtained by fitting to equation 1 of the extension-relaxation traces \(x(t)\), similar to the ones in Fig.5a and Supplementary Figure S4a, but measured at \(F=30\) pN and in the presence of several ActD concentrations. The fit (green line) through \(F=30\) pN and in the presence of several ActD concentrations. The fit (green line) through the on rates (green points) corresponds to the best-fit value \(k_a(30\text{pN}) = (8.4 \pm 0.8) \times 10^3 \text{ M}^{-1}\text{s}^{-1}\), which agrees well with the \(k_a\) obtained at 30 pN from the experiments done in the presence of 500 nM ActD (Figure 5d). The off rates (red points) exhibit no [ActD]-dependence within error, yielding an average \(k_{off}(30\text{pN})=(0.001\pm0.0005) \text{ s}^{-1}\) consistent with the \(k_{off}(30\text{pN})\) value obtained in other experiments (see Figure 5c).
Supplementary Figure S7: Direct observation of off rates in washing experiments. After extensions were obtained at different constant forces while flowing 500nM ActD through the flow cell as described in Figure 5a, ActD was washed away with buffer flow. Extensions obtained (open circles) and exponential fits (solid curves) at different constant forces (11 pN- violet, 21 pN- blue, 31 pN- green and 41 pN- red) while washing away ActD are shown in this figure. The directly measures off rates from these fits (brown points in Figure 5c) are in excellent agreement with the off rates calculated by methods described in the manuscript (red points in Figure 5c).
Supplementary Figure S8: Concentration dependence of kinetic critical force validates the intercalation kinetics of ActD. Measurement of the kinetically determined critical force $F_k([\text{ActD}])$, which is the force that promotes major intercalation of ActD into dsDNA at $v=100$ nm/s. The data points shown are the force values corresponding to the abrupt decrease of the slope of $F(x)$ in Figure 3b, defined as $F_k$. The line is the fit of these data to equation [S8.1] with the best fitting parameters: $x_{on}= 0.30 \pm 0.05$ nm/bp and $[\text{ActD}^*] = 8 \pm 1$ µM.

$$F_k([\text{ActD}],v) = \frac{k_BT}{x_{on}} \ln \left( \frac{[\text{ActD}]}{[\text{ActD}^*]} \right)$$  \hspace{1cm} [S8.1]

where

$$[\text{ActD}^*] = \frac{v}{N\Delta x_{eq} k_a(0)} ,$$  \hspace{1cm} [S8.2]

has the meaning of the ActD concentration at which no force is required for complete dsDNA saturation with ActD, i.e. $F_k([\text{ActD}^*])=0$, on the time scale of stretching $\tau_{str} \sim N\Delta x_{eq}/v$. Here $N\Delta x_{eq} \sim 10\mu$m is the total DNA elongation during stretching, ($N \sim 5 \times 10^4$ is the number of base pairs in the case of $\lambda$-DNA, and $\Delta x_{eq} \sim 0.2$ nm is equilibrium DNA elongation per base pair due to individual ActD intercalation event). Equation [S8.1] was obtained from the condition

$$k_{on}(F, v, [\text{ActD}]) \approx k_{str} ,$$

such that the ActD-DNA on rate, $k_{on}(F) = k_a(0) [\text{ActD}] \exp(F x_{on}/kT)$,
become similar to the stretching rate, \( k_{str} \sim \frac{1}{\tau_{str}} \) at force \( F_k \). The fitted \( x_{on} = 0.30 \pm 0.05 \) nm is in agreement with the measurements \( x_{on} = 0.33 \pm 0.03 \) nm and \( x_{on} = 0.26 \pm 0.05 \) nm obtained by the analysis of measured \( k_{on}(F) \) in Figure 5c and in Supplementary Figure S5b, respectively. Also, saturation of DNA stretching curves at \( \nu = 100 \) nm/s is reached at \([\text{ActD}] \geq 4 \) \( \mu \)M consistent with the fitted value \([\text{ActD}^*] \approx 8 \) \( \mu \)M (Supplementary Figure S4). Using the latter quantity in conjunction with Eq. [S8.2], we obtain \( k_a(0) \approx 10^3 \) M\(^{-1}\)s\(^{-1}\), which is in very good agreement with the independent estimate of \( k_a(0) \) by zero force extrapolation of our measured \( k_a(F) \) in Figure 5d.
Supplementary Figure S9: DNA Structural dynamics that govern the binding of ActD. The DNA structural dynamics obtained using this study, show that DNA lengthening of $x_{on} \sim 0.3$ nm/bp during transition promotes the association of ActD, while it settles back to the length of $\Delta x_{eq} \sim 0.2$ nm/bp in the equilibrium bound state. In order to dissociate, the DNA length must increase again by $x_{off} \sim 0.1$ nm to go through the transition state. Due to DNA duplex destabilization accompanying these DNA length changes, the kinetics of ActD association and dissociation become much faster. This effect should also be observed for DNA destabilized by the helicase within transcription bubbles, resulting in the preferential targeting of cancer cells by ActD.

Supplementary References

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