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## Equilibrium and irreversible unzipping of DNA in a nanopore

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**Abstract.** – We study the unzipping dynamics of individual DNA hairpins using nanopore force spectroscopy at different voltage ramp rates and temperatures. At high ramp rates the critical unzipping voltage is proportional to  $\log \dot{V}$ , where  $\dot{V}$  is the voltage ramp. At low ramp values we observe a crossover to another regime with a weaker dependence on  $\dot{V}$ . Here we report on the dependence of these two regimes on temperature. Remarkably, the unzipping kinetics can be well described by a simple two-states model that predicts the existence of two asymptotic regimes: quasi-equilibrium unzipping at low-voltage ramps and irreversible unzipping at high ramp rates.

*Introduction.* – The dynamics of DNA unzipping and re-zipping is central for many biological processes such as replication, transcription and DNA repair [1]. The thermodynamic properties of base-pairing in DNA were determined by equilibrium measurements in bulk, and more recently single-molecule methods were applied to the study of basepair unzipping kinetics, and of the forces that enzymes apply in order to pull apart the two strands [2–7]. Nanopore force spectroscopy is an emerging single-molecule technique that allows one to test thousands of copies of the same molecule in short period of time [8]; unlike other single-molecule methods the polynucleotides do not need to be grafted to macroscopic beads or to surfaces. Instead, an electric field is used to guide the charged biopolymer through a narrow pore (nanopore) that can only admit a single strand of DNA (ssDNA) at a time.

The toxin  $\alpha$ -Hemolysin ( $\alpha$ -HL) has been extensively used to study the translocation dynamics of unstructured polynucleotides [9–11] and to unzip short double stranded DNA molecules [8, 12, 13]. Voltage drop across the nanopore applies force on the molecule in order to unzip it, and the unzipping time is precisely measured by the simultaneous recording of the residual ion current that flows through the blocked pore. Recently, we demonstrated that a dynamic voltage scheme can be used in this experiment to decouple the initial entry process of ssDNA overhang into the pore, from the subsequent unzipping kinetics. This gave us access to very small forces or small loading rates, and in particular to unzipping voltages much below the threshold potential required for the entry of the ssDNA to the pore ( $\sim 50$  mV). At high voltages or high loading rates (defined as the change in voltage over time,  $\dot{V} = dV/dt$ ), the data

suggests that unzipping is an irreversible transition with negligible backward (re-zipping) rate. However, at low voltages and in particular at low  $\dot{V}$  values, we observed systematic deviations from this approximation [8]. Here, we present a two-states model of the unzipping process that includes both forward and backwards transitions, followed by an irreversible escape from the nanopore. The model is then tested by an extensive experimental study of the temperature dependence of the unzipping kinetics of DNA hairpin molecules. Our results show that the unzipping process can be characterized in terms of two asymptotic regimes: 1) a quasi-equilibrium regime at small  $\dot{V}$  (low force) in which the hairpin in the pore fluctuates between closed and open states separated by a free energy barrier, and 2) a non-equilibrium regime at high  $\dot{V}$  (high force) in which re-zipping is negligible and the hairpin exits the pore immediately following unzipping. The temperature dependence in these two regimes follows a modified Kramers law [8,14–17], with voltage- and temperature-dependent barrier height and position.

*Materials and methods.* – PAGE-purified ssDNA and DNA hairpins (Eurogentec, San Diego, Cal.) were buffered in 10 mM Tris, 1 mM EDTA, pH 8.5 solution. Before each measurement the samples were heated to 75 °C for 10 min and then quenched to 4 °C. The hairpins consist of a single stranded overhang of 50 adenines and a 7, 9 or 10 bp double stranded part separated by a loop of 6 bases (4 bases in the case of hairpin 3), with the following sequences:

HP1: 5'-GCTCTGTTGCTCTCTCGCAACAGAGC(A)<sub>50</sub>,  
 HP2: 5'-CTCTGTTGCTCTCTCGCAACAGAG(A)<sub>50</sub>,  
 HP3: 5'-GTCGAACTTTTGTTCGAC(A)<sub>50</sub>.

The basic apparatus and experimental method for reconstituting the  $\alpha$ -HL channel in a horizontally supported planar bilayer were described earlier [18]. Temperature was maintained at 5.0, 10.0, 15.0 or 20.0  $\pm$  0.1 °C using a custom cell design [19]. The two chambers of the cell were filled with a 1 M KCl, 10 mM Tris-Cl buffer solution with a pH of 8.5. We used a patch-clamp amplifier to record the ionic current through the  $\alpha$ -HL channel. This signal was filtered using a 80 kHz low-pass 4-poles Butterworth filter and was digitized at 333 kHz/16 bits. Our acquisition system included a dynamic control of the voltage in order to apply any pattern of voltage after the entry of each DNA molecule into the pore [8,19]. This feature is particularly useful for studying the low-voltage kinetics of DNA in the pore.

The unzipping times for various voltage ramps and at different temperatures were measured using the following procedure. We threaded the single stranded overhang of the molecule in the pore by applying a voltage of 120 mV for a time  $t_d$ , which was set to be slightly longer than the most probable translocation time for 50 bases poly dA ssDNA, as determined from translocation experiments at the corresponding temperature [11,18]. Immediately following threading the molecule was held in the pore at a low voltage of  $V_0 = 20$  mV, during  $t_h = 500 \mu\text{s}$ . Then a ramp of voltage of slope  $\dot{V}$  was applied. Finally, in order to clear the pore of any remaining DNA, we applied a voltage of 120 mV during 1 ms (DNA that remained in the pore at the beginning of the pore clearing voltage was not counted [8]).

A typical unzipping trace measured using a ramp of 11 V/s is shown on the left-hand panel of fig. 1. The unzipping moment is clearly discerned as an abrupt jump in the measured ion current at  $t_U \simeq 8.5$  ms, which corresponds to an unzipping voltage of  $\sim 125$  mV, using  $V_U = \dot{V}t_U + V_0$ . Three typical unzipping distributions, each compiled from  $\sim 1000$  unzipping events are shown in the middle panel, at 12 V/s, 4.66 V/s and 0.61 V/s for the top, center and bottom distributions respectively. From each distribution we extracted the critical unzipping voltage  $V_C$  (most probable value of  $V_U$ ). The dependence of  $V_C$  on the voltage ramp is shown in fig. 1 (right panel) for 7, 9 and 10 bp hairpins. Figure 2 displays the dependence of  $V_C$  on the voltage ramp for the 10 bp hairpin at different temperatures.

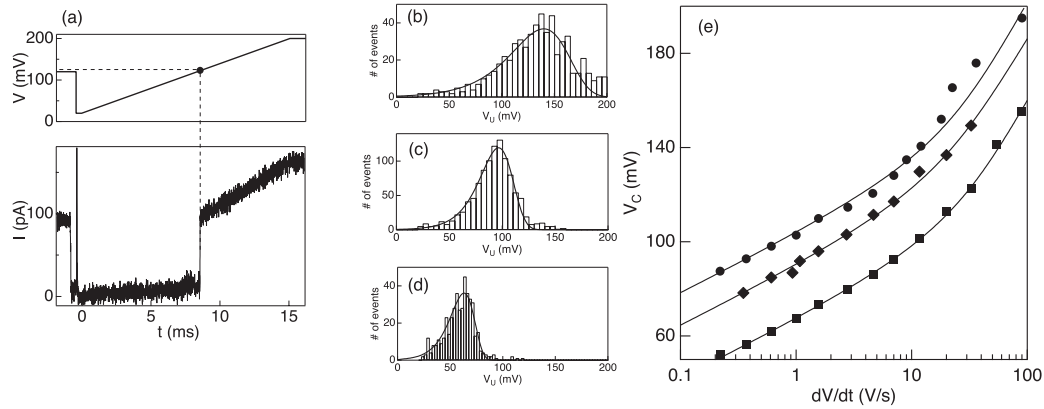


Fig. 1 – Left: the applied voltage (top) and measured current (bottom) as a function of time for a typical unzipping event. Center: typical unzipping voltage distributions for a ramp slope of: (b) 12 V/s at 15°C for 10 bp hairpin HP1, (c) 4.66 V/s at 15°C for 9 bp hairpin HP2 and (d) 0.61 V/s at 15°C for 7 bp hairpin HP3. Solid lines were calculated using eq. (5). Right (e): the critical unzipping voltage for the ramp for three different hairpins: HP1 (10 bp, circles), HP2 (9 bp, diamonds) and HP3 (7 bp, squares) as a function of the ramp level.

*Results.* – Going back to fig. 1 (right panel) we can clearly distinguish two regimes in this semi-log plot, for the three hairpin molecules we tested: at low ramp values (*e.g.* below  $\sim 5$  V/s) the curves approach a uniform slope of  $\sim 10.7$  mV, and at high ramp values they curve up and approach a slope of  $\sim 40$  mV. As expected, the curves corresponding to the

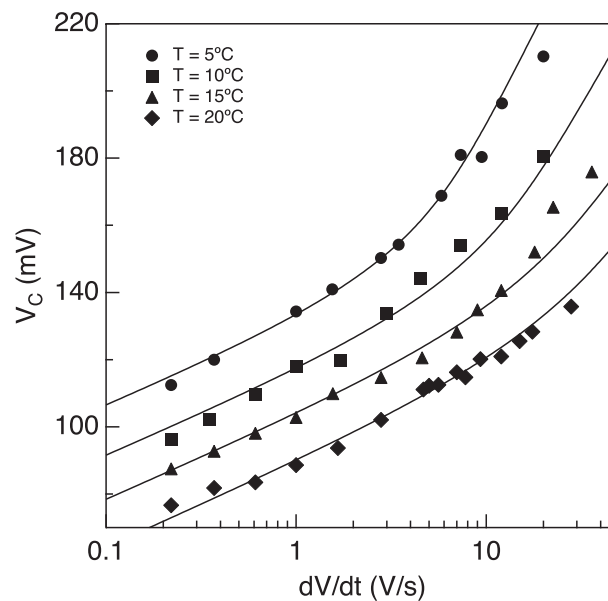


Fig. 2 – Critical unzipping voltages,  $V_C$ , measured for the 10 bp hairpin HP1 as a function of ramp slope at different temperatures (5, 10, 15 and 20°C). Solid lines were determined using eq. (6).

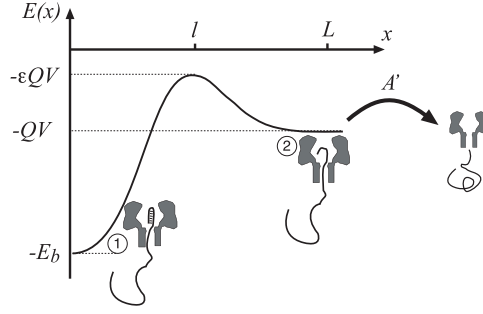


Fig. 3 – Schematic energy landscape of the two-states model developed.

three different hairpins are organized in ascending order according to their length, with a shift of nearly 15 mV for each additional basepair in the hairpin. Using a modified Kramers model, following [16], one can obtain the relation between  $V_C$  and  $\dot{V}$ :  $V_C = V_\beta \ln\left(\frac{\dot{V}\tau_0}{V_\beta}\right)$  for the simplified case of an unzipping transition across a free energy barrier, with no backward rate [8]. This approximation yields a single regime with a uniform slope (on the semi-log plot) defined as  $V_\beta = kT/Q$ , where  $Q$  is a constant with the dimensions of electric charge. To account for the two regimes observed in fig. 1 we have extended the model to include backward (re-zipping) processes as described in detail below.

In order to determine the temperature dependence of the unzipping kinetics we repeated our measurements using HP1 at  $T = 5, 10, 15,$  and  $20^\circ\text{C}$ . The results are shown in fig. 2. As expected, increasing the temperature decreases the critical voltage required for unzipping. We note, however, that the effect of the temperature is not the same in the two regimes of the curves: while at low ramp values the slope is almost unaffected by temperature, at the high ramp values the low temperature data display a steeper ramp dependence.

To explain this behavior we introduce a two-states model, which takes into account the characteristics of our system. Figure 3 displays a schematic view of the energy landscape along the pore axis under applied voltage. State 1, located at  $x = 0$ , represents the DNA threaded in the pore with the hairpin closed. State 2 is located at  $x = L$  (the length of the pore) and represents the hairpin in an open state but with the molecule still inside the pore. A tentative picture for the transient configuration is a hairpin with broken bonds between (all or some of) the bases, that is pulled a distance  $x = l$  into the pore. From state 2, the DNA exits the pore at a rate  $A'$ . In this model, we have made two simplifying assumptions: 1) we neglect the hairpin entropy, which implies that the free energy difference between state 1 and 2 is equal to the energy barrier height at  $V = 0$ . 2) We neglect the voltage dependence of  $A'$ . We note that these approximations do not impact the asymptotic behavior of the unzipping voltage (at very high or very low  $\dot{V}$ ), which are the main focus of this paper. A more detailed study that specifically include the voltage dependence of  $A'$  and the hairpin entropy is left for a future publication.

The rate equations for this model can be written as

$$\frac{dC_1}{dt} = -P_{12}C_1 + P_{21}C_2, \quad (1)$$

$$\frac{dC_2}{dt} = P_{12}C_1 - (A' + P_{21})C_2, \quad (2)$$

where  $C_1$  and  $C_2$  are the concentrations of states 1 and 2,  $P_{12}$  and  $P_{21}$  are the probabilities per unit time of going from state 1 to 2 and from state 2 to 1, respectively, and  $A'$  is the rate

at which molecules leave the pore from state 2. The application of voltage reduces the barrier  $E_b \rightarrow E_b - \varepsilon QV$  and shifts the energy of state 2 downwards by  $-QV$ , thus yielding  $P_{12} = \tau_0^{-1} e^{\varepsilon v}$  and  $P_{21} = A e^{-(1-\varepsilon)v}$ , where  $\tau_0 = A^{-1} e^{E_b/k_B T}$ ,  $\varepsilon \equiv \frac{L}{L}$ ,  $v \equiv \frac{QV}{k_B T} \equiv \frac{V}{V_\beta}$  and  $A$  is the attempt rate for the hairpin opening. The rate equations (1) and (2) can be recast into the form

$$\tau_0 \frac{dC_1}{dt} = -e^{\varepsilon v} C_1 + e^{-(1-\varepsilon)v} C_2', \quad (3)$$

$$\tau_0 e^{-\varepsilon_b} \frac{dC_2'}{dt} = e^{\varepsilon v} C_1 - (\alpha + e^{-(1-\varepsilon)v}) C_2', \quad (4)$$

where  $\varepsilon_b \equiv E_b/k_B T$ ,  $\alpha \equiv A'/A$  and  $C_2' \equiv C_2 e^{\varepsilon_b}$ .

In general, eqs. (3) and (4) can be solved analytically for the case of stationary voltage ( $\dot{V} = 0$ ), but not for a non-vanishing ramp. However, an approximate solution for  $\dot{V} \neq 0$  can be constructed in the limit  $e^{-\varepsilon_b} \ll 1$  and  $A'\tau_0 \gg 1$  in which the term on the left-hand side of eq. (4) vanishes, and one obtains the following distribution of unzipping voltages for a given ramp rate [20]:

$$P(v) = P_0 \frac{g(v)}{\alpha} \exp \left[ -\frac{\int_{v_0}^v g(x) dx}{v'} \right], \quad \text{where } g(v) = \frac{\alpha e^v}{1 + \alpha e^{v(1-\varepsilon)}}, \quad (5)$$

where  $v' = \dot{V}\tau_0/V_\beta$  and  $v_0 = V_0/V_\beta$ . The critical voltage  $V_C$  is defined as the voltage at the maximum of  $P(v)$  and is determined by the relation

$$\dot{V} = \frac{V_\beta}{\tau_0} \frac{\alpha e^{\frac{V_C}{V_\beta}}}{1 + \varepsilon \alpha e^{\frac{V_C}{V_\beta}(1-\varepsilon)}}. \quad (6)$$

This function cannot be inverted and thus we cannot derive an explicit expression for  $V_C(\dot{V})$  to compare with our experimental data (figs. 2 and 3). However, one can derive the asymptotic limits of eq. (6). At low ramp values ( $\dot{V} \rightarrow 0$ ) the critical voltage behaves as  $V_\beta \ln \left( \frac{\dot{V}\tau_0}{V_\beta \alpha} \right)$  (slope =  $V_\beta$ ). At high ramp values ( $\dot{V} \rightarrow +\infty$ ) the critical voltage approaches  $\frac{V_\beta}{\varepsilon} \ln \left( \frac{\dot{V}\tau_0 \varepsilon}{V_\beta} \right)$  (slope =  $\frac{V_\beta}{\varepsilon}$ ).

In order to fit our data (figs. 1 (right panel)) and 2) with eq. (6) we used the asymptotic limits described above to determine  $V_\beta$  and  $\varepsilon$  at each given temperature. Our results are

TABLE I – Summary of the parameters obtained from fitting the data shown in fig. 1(e) and fig. 2 using eq. (6).

Hairpin	$T$ ( $^\circ\text{C}$ )	$V_\beta$ (mV)	$\varepsilon$	$\tau_0$ (ms)	$\alpha$ ( $\times 10^{-4}$ )
HP1	5	10.31	0.21	230	0.7
HP1	10	10.49	0.24	120	1.9
HP1	15	10.68	0.28	75	4.6
HP1	20	10.86	0.29	45	10.9
HP2	15	10.68	0.28	65	4.6
HP3	15	10.68	0.28	25	4.6

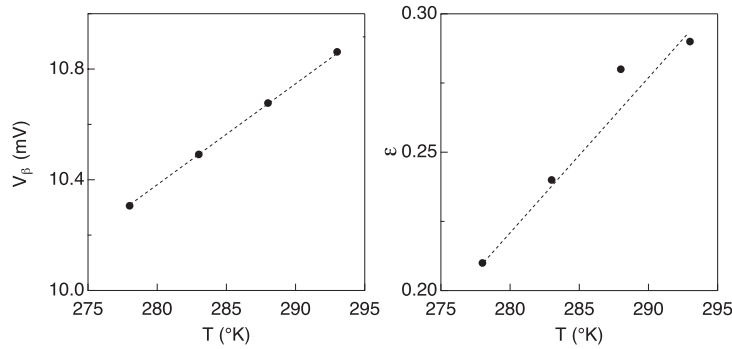


Fig. 4 – The dependence of  $V_\beta$  and  $\varepsilon$  on the temperature, determined by an asymptotic expansion of eq. (6) (see fig. 2). Dashed lines are linear regression fits.

displayed in fig. 4 and table I. We note that  $V_\beta$  follows a linear dependence on temperature,  $kT/Q$ , where  $Q = 2.24e$  ( $e$  is the elementary charge) can be interpreted as an effective charge of DNA inside the pore, on which the electric field is applied. This value is much smaller than the stoichiometric value of the charge,  $12e$ , as expected from MD simulations that show enhanced counter-ion condensation on DNA inside the pore [21]. The parameter  $\varepsilon$  increases linearly with temperature, in the range 0.2–0.3, showing that the distance to the metastable state (the barrier) increases with temperature.

Using the values of  $V_\beta$  and  $\varepsilon$  discussed above, we attempted to determine the other two parameters,  $\tau_0$  and  $\alpha$  (see eq. (6)). However, while  $V_\beta$  and  $\varepsilon$  can be reliably determined, the uncertainty in the determination of  $\tau_0$  and  $\alpha$  is high. Going back to eq. (6) we note that the dependence of  $\dot{V}$  on  $\tau_0$  and  $\alpha$  is weak as compared to its dependence on  $V_\beta$  and  $\varepsilon$  (linear *vs.* exponential dependence, respectively). This precluded us from formally fitting our data to eq. (6), with all four free parameters. Nevertheless, the best fit to our data (shown as solid lines) yielded an exponential dependence of  $\tau_0$  on  $1/T$ , as expected, with a reasonable value for barrier height  $E_b = 30k_B T_0$  ( $T_0 = 300$  K). Due to the small temperature range in our experiments and the difficulties in determining  $\tau_0$  we expect a large error bar on its value.

*Discussion and conclusions.* – The two-states model introduced in this work predicts the existence of two regimes in the ramp unzipping, as seen in the experiment. At low ramp rates, the slope of the experimental data is  $V_\beta$ . In this regime the time scale of the hairpin re-closing (going from state 2 to state 1) is much shorter than the time scale of leaving the pore and the hairpin undergoes many transitions between the two states 1 and 2 before finally leaving the pore. In the high ramp regime the slope is  $V_\beta/\varepsilon$ . Here the system does not have enough time to equilibrate, since the characteristic time scale to leave the pore is much shorter than the re-closing time (the barrier that the molecule must cross to re-close becomes larger as the voltage increases). The observed temperature effects can be reproduced by the model by introducing temperature-dependent barrier location  $\varepsilon(T)$ . Even though more rigorous theoretical models of the unzipping kinetics have been proposed [22, 23], the advantage of the simplified description presented here is that it provides us with a simple physical picture of the unzipping process. The results of the temperature dependence studies are particularly significant: as expected, we found that  $V_\beta$  depends linearly on temperature,  $\varepsilon$  increases linearly with temperature, and that  $\tau_0$  decays exponentially with  $1/T$  yielding an energy barrier, which is close to the equilibrium barrier calculated using the *mfold* server (16.4 kcal/mol) [24]. The measurements reported here were also performed using a step voltage scheme instead of a

ramp. We found that in the range of 30–200 mV our step data can be fitted with the present model, yielding the same parameters described for the ramp unzipping for each temperature (data not shown). We note however, that the step unzipping produced higher systematic experimental errors, especially at the two extremes (very low and very high voltages), and that overall we found the ramp unzipping to be more practical.

\* \* \*

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