

Kinetic barriers in RNA unzipping

A. Imparato^a and L. Peliti^b

Dipartimento di Scienze Fisiche and Unità INFN, Università “Federico II”, Complesso Universitario di Monte S. Angelo, 80126 Napoli, Italy

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Abstract. We consider a simple model for the unfolding of RNA tertiary structure under dynamic loading. The opening of such a structure is regarded as a two step process, each corresponding to the overcoming of a single energy barrier. The resulting two-barrier energy landscape accounts for the dependence of the unfolding kinetics on the pulling rate. Furthermore at intermediate force, the two barriers cannot be distinguished by the analysis of the opening kinetic, which turns out to be dominated by a single macro-barrier, whose properties depend non-trivially on the two single barriers. Our results suggest that in pulling experiments on RNA molecule containing tertiary structures, the details of the single kinetic barriers can only be obtained using a low pulling rate value, or in the high force regime.

PACS. 87.14.Gg DNA, RNA – 82.37.-j Single molecule kinetics

1 Introduction

The study of structural properties of biological molecules has received a boost by the introduction of techniques allowing for the manipulation of single molecules. For example, the study of folding and unfolding of nucleic acids can now be performed by applying a controlled force on the free end of a single strain of a molecule. In this situation, the opening of the Watson-Crick pairs leads to what has been called the *unzipping* of the molecule. Unzipping in DNA is similar to some steps involved in DNA replication and in its translation into mRNA, and has been the subject of several theoretical and experimental investigations [1,2]. On the other hand, RNA unzipping exhibits further complications, since a given single stranded RNA molecule can exhibit a complex secondary structure (matching pattern between complementary bases) and tertiary structure (three-dimensional conformation). Thus, single-molecule unzipping experiment can yield information on the secondary structure of RNA molecules [3–7]. In reference [5] in particular, the role of the secondary structure intermediates in the folding/unfolding experiments is discussed, and it is shown that such intermediates can be responsible for the slowing down of the kinetics. Moreover, the response of complex RNA structures to applied mechanical forces can be analogous to the responses of RNA during translation or export from the nucleus [4]. However, in such cases, the tertiary RNA structure plays an

important role. It is commonly believed that the breaking of RNA tertiary structures or their formation are the time limiting processes in the molecule unfolding or refolding, respectively [3,8]. In the unzipping experiments, tertiary contacts may lead to the appearance of kinetic barriers where the unzipping process momentarily stops [3,4]. In this situation, overcoming these barriers bears some analogy with the breakup of molecular adhesion studied in a number of experiments [9,10] which have been recently reanalyzed theoretically [11–13]. In the present paper, we wish to understand some features of the experiments on RNA unzipping in the light of this theory, in order to highlight what information one is able to collect from the unzipping kinetics on the position and the height of the barriers due to the tertiary structure. Although in the case of the folding of large complex RNA molecules, the formation of kinetically trapped intermediates in the secondary structure can play an important role in the slowing down of the process [14–16], here we will specifically consider the role of the tertiary structures in the unzipping experiments. Such role was stressed in references [3,4], where the mechanical unfolding of RNA molecules were performed. In those experiments the tertiary contacts could be removed by changing the solution the RNA was immersed in. The removal of the tertiary structures corresponded to the disappearing of the kinetic barriers, and furthermore the folding/unfolding processes became reversible. This kind of experiments indicate that the tertiary structures are responsible for the kinetic arrest of the structural rearrangement of RNA molecules under tension, although the tertiary structures are much more brittle compared to

^a e-mail: imparato@na.infn.it

^b Associato INFN, Sezione di Napoli

the secondary ones, i.e. they break in consequence of small deformations.

2 The model

A typical unfolding experiment consists in holding one molecule's free end in an optical trap while the other free end is pulled at constant velocity [3,4]. This induces a pulling force on the molecule that increases linearly with time $f(t) = rt$, where r is the loading rate. The typical output of such experiment is a force-extension curve where the monotonic increase of the force is interrupted by a number of plateaus revealing the unfolding of portions of the molecule.

The unfolding of the molecule is a stochastic process, which depends on the pulling force rate r , the actual value of the pulling force and the microscopic details of the molecule. Therefore the unfolding of an RNA molecule, i.e., the successive breaking of its molecular links, can be viewed as a succession of thermally activated escape processes over a set of energy barriers, each representing one or more molecular links [8,14,15]. Within this picture, the resulting energy landscape can be considered one-dimensional, since the experimental set-up singles out a well-defined direction, which is the pulling direction. The landscape energy barriers are thus located at increasing distance along the pulling direction. This experiment can be repeated for several values of r , in such a way that the different force-extension curves can be mapped onto a single curve of the breaking force as a function of the loading rate. From this curve, much information on the microscopic details of the molecular bonds can be obtained [9,10]: in particular the typical length and energy of such bonds. Another quantity, which can be obtained by the force-extension characteristics, is the fraction ϕ of molecules which remain folded as a function of the time or the force, and which also depends on the parameter r . Sampling the fraction of folded molecules at different times provides a more direct insight into the kinetic barriers which slow down the unfolding process. Using Evans' results for the case of pulling experiments on a single molecular link [10], which can be represented by a single kinetic barriers of height ΔE and position Δx along the reaction coordinate, the fraction $\phi(t)$ of bound links at time t is given by

$$\begin{aligned} \phi(t) &= \exp \left[- \int_0^t dt' \omega_0 e^{-\beta(\Delta E - rt' \Delta x)} \right] \\ &= \exp \left[- \frac{\omega_0}{\beta r \Delta x} e^{-\beta \Delta E} (e^{\beta r t \Delta x} - 1) \right], \end{aligned} \quad (1)$$

where ω_0 is the attempt frequency, which depends on the microscopic details of molecular linkage, and $\beta = 1/k_B T$.

In the large force regime ($f \geq 10$ pN), one expects that the quantity $q(f)$ defined as

$$q(f) = \ln \left[r \ln \frac{1}{\phi(t)} \right] \Big|_{t=f/r}, \quad (2)$$

will be a linear function of f :

$$q(f) \simeq \ln \left(\frac{\omega_0}{\beta \Delta x} \right) - \beta \Delta E + \beta f \Delta x, \quad (3)$$

and will exhibit no dependence on r .

This expression has been exploited by Liphardt et al. [3] to characterize the tertiary structure of an RNA molecule: by mechanically pulling on the P5abc domain of the *Tetrahymena thermophila* ribozyme, the fraction of folded molecules as a function of the force (time) has been determined. The authors then make the hypothesis that the tertiary structure can be described as a single kinetic barrier, which hinders the molecule from unzipping. Using equation (3), they give an estimate for the two characteristic parameters of the barrier, i.e. the zero-force transition rate, defined as

$$k_0 = \omega_0 \exp(-\beta \Delta E). \quad (4)$$

and the barrier position along the reaction coordinate Δx (in that case and in the following in this paper, the elongation of the molecule needed to break the bond). However, the data for $q(f)$ showed in that paper, exhibit a dependence on r even if not distinct, in contrast with equations (2) and (3).

In the present paper we will argue that at least another kinetic barrier has to be considered in the energy landscape of the tertiary structure in order to account for the dependence of $q(f)$ on r . Furthermore, in a recent paper, Bartolo et al. [13] showed that in the more complicated case of several molecular links, which can be represented by a set of N kinetic barriers along a one-dimensional unbinding path, the unbinding force plotted as a function of logarithm of the pulling rate ($\ln r$) appears as a succession of straight lines whose slopes are given by the distances between the adjacent maxima and the minima of the energy landscape. Thus, we expect that for the simple two-barrier energy landscape here considered, the plot of q as a function of $f = rt$ will exhibit more than one straight line, each corresponding to a different escape route from the folded to the unfolded state.

Basically we assume that the tertiary structures of a RNA molecule can be described by more than one energy barrier with different width and height: the simplest case is the one depicted in Figure 1, where a landscape $E(x)$ characterized by two barriers is responsible for the slowing of the unfolding process, and the state $x = 0$ corresponds to the unperturbed structure. The opening of the tertiary structure can thus be regarded as a two-step process: when the molecule extension is of the order of x_A , as a consequence of thermal denaturation or pulling force, a first set of tertiary interactions are broken, and the system jumps into the local energy minimum at x_a . Later, as the length x_B is reached, the remaining tertiary interactions break down. The choice of a two-barrier landscape has been also suggested to us by the following experimental observation: in RNA pulling experiments, a small but finite fraction of molecules under dynamic loading unfolds with two successive rips [3], revealing the existence of an

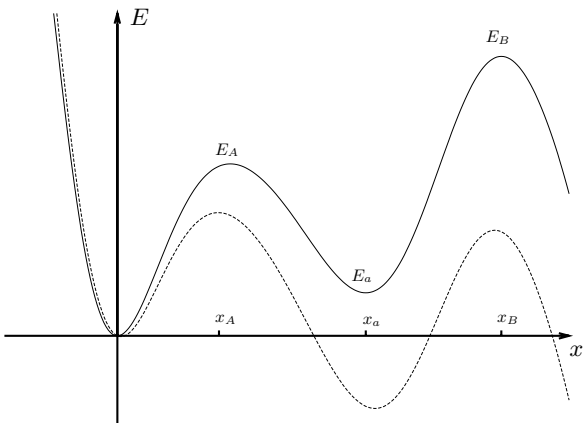


Fig. 1. Schematic plot of one-dimensional energy landscape with two energy barriers. The full line corresponds to the landscape with no external force applied $E(x)$, while the dotted curve corresponds to the time-dependent landscape $E(x) - rtx$ at $t > t_A$ (see text and Eq. (5) for the definition of t_A).

intermediate state between the completely folded and the unfolded ones.

When force is applied, the energy landscape changes as $E(x, t) = E(x) - rtx$, so that the outermost barrier height decreases faster than the inner one: the unfolding kinetics is thus characterized by two crossover times which will be respectively indicated with t_A and t_a in the following. For times longer than t_A , the inner barrier becomes the dominant one and is mainly responsible for the slowing down of the unfolding process with respect to the $f = 0$ case. Eventually, at times longer than t_a (with $t_a > t_A$), the well (x_a, E_a) disappears and the unfolding simply results from the overcoming of the first barrier (x_A, E_A) . If we make the further assumption that the energy barriers and wells sketched in Figure 1 are sharp, so that the barrier and well positions remain essentially constant with time, the crossover time t_A is given by

$$t_A = \frac{E_B - E_A}{r(x_B - x_A)}, \quad (5)$$

and t_a is given by

$$t_a = \frac{E_B - E_a}{r(x_B - x_a)}. \quad (6)$$

The last equation also defines a crossover force $f_a = r \cdot t_a$. Using a Kramer formalism, and the notations shown in Figure 1, we can write the instantaneous rate for the transition from one of the two minima $(x = 0, x_a)$ over the two corresponding energy barriers $(x = x_A, x_B)$:

$$k_{0 \rightarrow a} = \omega_0 \exp[-\beta(E_A - f(t)x_A)], \quad (7)$$

$$k_{a \rightarrow 0} = \omega_0 \exp[-\beta(E_A - E_a - f(t)(x_A - x_a))], \quad (8)$$

$$k_{a \rightarrow \infty} = \omega_0 \exp[-\beta(E_B - E_a - f(t)(x_B - x_a))], \quad (9)$$

where $x = \infty$ indicates the completely unfolded state. We assume furthermore $k_{\infty \rightarrow 0, a} = 0$, i.e., once unfolded the

system never folds back, as observed experimentally [3]. Let $p_0(t)$ and $p_a(t)$ be the probabilities that the system is in the state 0 or a , respectively. The time evolution of this quantities is described by the following differential equation system

$$\dot{p}_0 = -k_{0 \rightarrow a}p_0 + \theta(t_a - t)k_{a \rightarrow 0}p_a, \quad (10)$$

$$\begin{aligned} \dot{p}_a = & \theta(t_a - t)k_{0 \rightarrow a}p_0 - \theta(t_a - t)(k_{a \rightarrow 0} + k_{a \rightarrow \infty})p_a \\ & - \theta(t - t_a)\omega_0, \end{aligned} \quad (11)$$

where $\theta(t)$ is the Heaviside step function. The step functions θ have been included in equations (10) and (11) in order to take into account the disappearing of the well (x_a, E_a) from the system energy landscape at $t = t_a$. It is worth to note that at such crossover time, a significant fraction of molecules might be accumulated in the $x = x_a$ state, as a result of the system evolution at previous times. Thus we assume that at time $t > t_a$, the escape rate of the molecules which are still in the state $x = x_a$, is determined by the molecular attempt frequency ω_0 alone. For a given set of characteristic parameter

$$\mathcal{S} = \{\omega_0, x_A, E_A, x_a, E_a, x_B, E_B\},$$

given the initial values $p_0(0) = 1$ and $p_a(0) = 0$, such a system can be solved numerically. As mentioned above, the molecule never folds back, once it has been completely unfolded, therefore the quantity

$$\phi(t) = p_0(t) + p_a(t) \quad (12)$$

defines the probability that the system is *still* folded at time t , either completely in the $x = 0$ state or partially in the $x = a$ state.

In order to obtain a reliable estimate of the parameter set \mathcal{S} , we consider the results of the above cited experiment: applying equation (3) to the openings of a simple RNA molecule tertiary structure, a zero-force transition rate $k_0 \simeq 2 \times 10^{-4} \text{ s}^{-1}$ and a difference between the unfolded tertiary structure length and its transition (breaking) length $\Delta x \simeq 1.6 \text{ nm}$ have been obtained [3]. The zero-force transition rate $k_0 = 2 \times 10^{-4} \text{ s}^{-1}$ is related to the attempt frequency ω_0 and to the overall energy barrier ΔE of the single barrier picture via equation (4). In order for the tertiary structure to be the dominant impedance to the molecule unfolding, the involved energy barriers have to be greater than the well known RNA base pair energies, which are of the order of a few $k_B T$. Thus, if we suppose that the energy barrier ΔE is of the order $\Delta E \simeq 10 k_B T$, from the above cited result and from equation (4), we obtain $\omega_0 \simeq 4.4 \text{ s}^{-1}$. Our estimate for the tertiary structure energy is in agreement with the values shown in [17], where combining numerical computations with experimental techniques, the energy of tertiary interactions in a simple RNA molecules was found to range between $6 k_B T$ and $13 k_B T$. On the other hand, a direct measurement of the attempt frequency ω_0 in folding/unfolding experiments is rather difficult. An indirect estimate can be obtained by pulling the RNA molecule at constant force, when the force value is within an interval of a few pNs

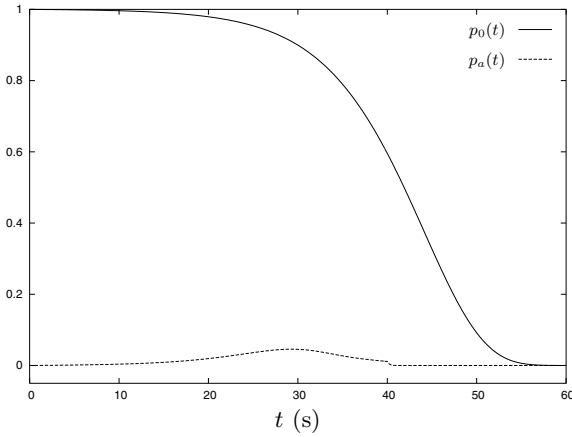


Fig. 2. Probabilities p_0 and p_a as functions of the time, obtained by numerical integration of equations (10) and (11) with $r = 1$ pN/s.

around the unfolding force. The molecule then hops back and forth between the folded and unfolded state with a frequency between 0.05 s^{-1} and 20 s^{-1} [3], which depends on the actual value of the force. It can be assumed that, at the unfolding force, the effective energy barrier vanishes and the hopping rate yields a rough estimation of the microscopic attempt rate. Thus our estimate for the attempt frequency ω_0 is in agreement with those experimental results.

Here we take $x_A = 0.6$ nm, $x_a = 0.8$ nm, and $x_B = 1.8$ nm: compared to the above cited result, our choice corresponds to two successive openings which occur at elongations whose sum is equal to the single step picture elongation $\Delta x = 1.6$ nm. The set of parameter values that we will consider in the following is thus given by

$$\mathcal{S} = \{ \omega_0 = 4.4 \text{ s}^{-1}, x_A = 0.6 \text{ nm}, E_A = 10 k_B T, \\ x_a = 0.8 \text{ nm}, E_a = 6 k_B T, x_B = 1.8 \text{ nm}, E_B = 16 k_B T \}, \quad (13)$$

with $T = 300$ K. Note that with this choice for the energy barrier height, after the first opening, the system has to overcome another barrier of relative height $E_B - E_a = E_A$ in order for the second opening to occur. The two probabilities $p_0(t)$ and $p_a(t)$, obtained by numerical integration of equations (10) and (11) with $r = 1$ pN/s, are shown in Figure 2.

3 Results

In Figure 3 the function q , as defined by equation (2), is plotted as a function of f for the energy landscape represented in Figure 1, with the set of parameter values \mathcal{S} given by equation (13), and for different values of the loading rate r : the behavior of such function turns out to be dependent on both the force range and the loading rate. For all the value of r here considered, in the large force regime $f > f_a$, all the curves collapse on a single scaling curve, which corresponds to the escape from the innermost

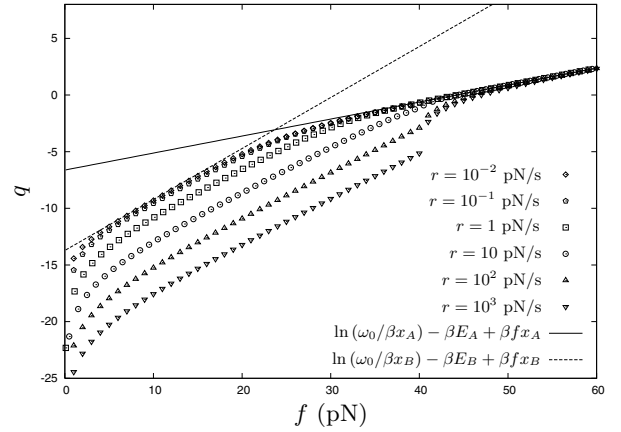


Fig. 3. Plot of q as a function of f , as defined by equation (2), for different values of r . At large force, for each value of the loading rate r , the curves all collapse on a single scaling function which corresponds to the escape from the innermost barrier (x_A, E_A) (full line). For small values of r , and in the small force regime, the curves also converge to a function which corresponds to the escape from the outermost barrier (x_B, E_B) (dashed line): in this limit the structure of the inner part of the energy landscape does not affect the behavior of the function $q(f)$.

barrier E_A at x_A , i.e., after the outermost barrier has disappeared, the innermost barrier becomes the only obstacle for the unfolding of the molecule. On the other hand, for the smallest value of r here considered ($r = 10^{-2}$ pN/s), and at intermediate force values, $q(f)$ lies on a line given by equation (3) with $\Delta x = x_B$ and $\Delta E = E_B$.

These results are in agreement with those of the above mentioned work of Bartolo et al. [13], where the authors found out that slope of the breaking force as a function of $\ln r$ is equal to the position of the outermost barrier, in the small r regime. The same quantity has been found to be equal to the position of the innermost barrier at large r , if the relative height of the two barriers is similar, as in our case. It is worth to remark that in this cited work the single escape rate approximation has been used, i.e., it has been assumed that the mean escape time from each of the landscape barrier is constant. In the present work we do not use such approximation, and thus our results can be considered more general of those contained in [13].

The plot in Figure 3, indicates that the behavior of $q(f)$, in the intermediate force range, strongly depends on the value of the pulling rate r . By using linear fits, we find that for all the curves with $r \geq 1$ pN/s, in the force range $10 \text{ pN} \lesssim f \lesssim f_a \simeq 40 \text{ pN}$, the slope of $q(f)$ is equal to $x_m = x_B - x_a + x_A = 1.6$ nm. This indicates that the two bonds here considered, behave as a single macro-bond whose typical length is x_m . In analogy with the single bond case, in the force range $10 \text{ pN} \lesssim f \lesssim f_a$, the quantity q can be written as

$$q(f, r) = \ln \left[\frac{k(r)}{\beta x_m} \right] + \beta f x_m, \quad (14)$$

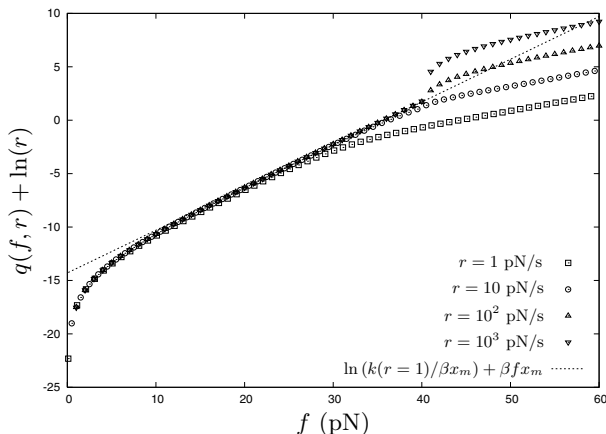


Fig. 4. Plot of $q(f, r) + \ln(r)$ as a function of f . The data all collapse on the same curve $r = 1$, in the force range $f \lesssim f_a$.

where we have explicitly taken into account the dependence on r , and where $k(r)$ is the zero-force transition rate of the macro-bond, which depends on the details of the energy landscape. Such a quantity decreases as the loading rate r increases, and it turns out to scale as $k(r) \propto r^{-1}$, as can be seen in Figure 4. Using qualitative arguments, Evans [10] has proposed that in a set of N identical molecular bonds in series, the zero force transition rate decreases as the inverse of N . In our case the inverse proportionality of the zero-force transition rate on r , appears to depend strongly on the model used here, and in particular on the form of equations (10) and (11) used to obtain the quantity $\phi(t)$. The dependence of q on the loading rate r , indicates that at a given force $f \leq f_a$, the fraction of molecules which remain in the state $x = 0$ before unfolding completely, increases with the loading rate r . This is also confirmed by a direct plot of the fraction of molecules that are in the ground state at the cross-over force f_a , as a function of r , see Figure 5. In other words as r increases, the molecule is more and more “frozen” in its ground folded state, and only after the outmost barrier disappears ($f = f_a$), the system unfolds in a way which is determined only by the inner barrier features.

The characteristics of the quantity $q(f, r)$ in the moderate force regime, i.e., the value of the slope $x_m = x_B - x_a + x_A$ and the scaling law of the zero-force transition rate $k(r)$, turn out to be fairly universal: we found the same results with different choices of parameter sets \mathcal{S} (data not shown). This is at variance with the results discussed in [11, 13] where the slope of the rupture force as a function of $\ln r$, in a given range of r , has been found to be characterized by a single length which corresponds to the relative position of the dominant barrier with respect to the adjacent energy minimum position. In other words, our results suggest that the unfolding process is not dominated by a single escape route over a well determinate barrier, but is rather controlled by the interactions between the two barriers, which determine a different escape route.

The analysis of the fraction of folded molecules $\phi(t)$ and its related function $q(f)$, that we have proposed so

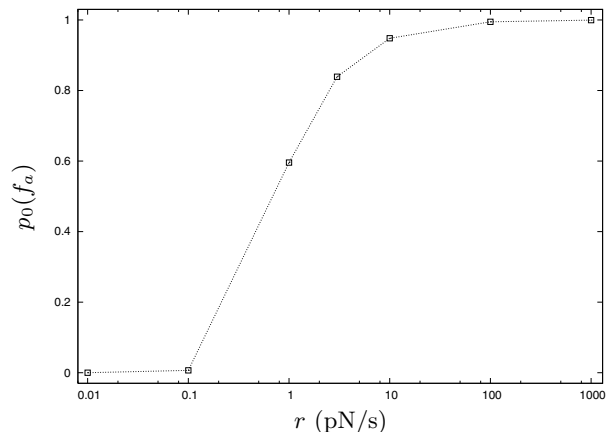


Fig. 5. Fraction of molecules $p_0(f_a)$, which “survives” in the state $x = 0$ at the crossover force f_a , as a function of r . The dotted line is a guide to the eye.

far, does not provide any estimate of the intermediate energy well E_a . In the following we propose a method to obtain the value of this quantity, once the fraction of (completely or partially) folded molecules ϕ and the fraction of molecules which are in the intermediate state p_a have been experimentally determined, as functions of the time (force). Let us consider the smallest value of the pulling rate we have used in this paper, $r = 10^{-2}$ pN/s. For this value, we have shown that

$$q(f, r = 10^{-2}) = \ln\left(\frac{\omega_0}{\beta x_B}\right) - \beta E_B + \beta f x_B, \quad (15)$$

which holds in low-to-moderate force range, see Figure 3. Using the definition of $q(f)$ as given by equation (2), we then obtain

$$\phi(t, r = 10^{-2}) = \exp\left[-\frac{\omega_0}{\beta r x_B} e^{-\beta(E_B - r t x_B)}\right], \quad (16)$$

and

$$\dot{\phi}(t, r = 10^{-2}) = -\omega_0 e^{-\beta(E_B - r t x_B)} \phi(t, r = 10^{-2}). \quad (17)$$

The last expression is expected to hold for sufficiently small values of the force. On the other hand, summing equation (10) and equation (11), we obtain, for $t < t_a$,

$$\dot{\phi}(t) = -k_{a \rightarrow \infty} p_a, \quad (18)$$

where $k_{a \rightarrow \infty}$ is given by equation (9). Putting together equation (17) and equation (18), yields

$$\frac{\phi(t, r = 10^{-2})}{p_a(t)} \Big|_{t=f/r} = e^{\beta(E_a - f x_a)}. \quad (19)$$

Thus we expect that, at low-to-moderate forces where the equality (16) holds, the ratio of ϕ to p_a , for small values of the pulling rate, is a linear function of the force, in a linear-log plot of the data. This is confirmed by Figure 6, where the ratio of ϕ/p_a , as obtained by numerical solution of

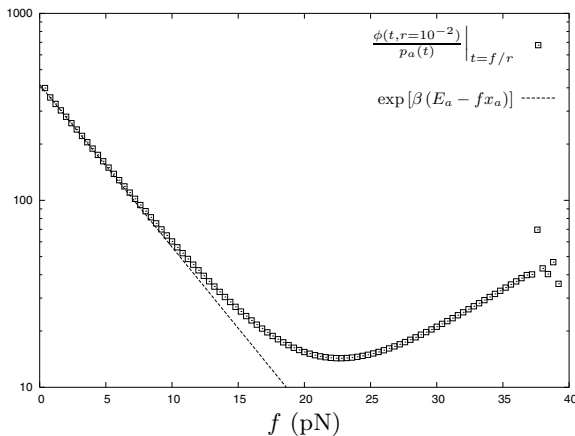


Fig. 6. Ratio of folded molecule fraction ϕ to the fraction p_a of molecules in the state $x = x_a$, as a function of the force f , with $r = 10^{-2}$ pN/s. The dashed line corresponds to the expected function in the small force regime, as given by equation (19).

equations (10) and (11) with $r = 10^{-2}$ pN/s, is plotted as a function of f . From a linear fit of the relative experimental data, it might then be possible to obtain estimates for x_a and E_a .

The behaviour of the model here proposed appears to be rather robust with respect to changes in the values of the parameters.

It is clear that, e.g., an increase in ω_0 as well as a (slight) decrease in the barrier heights would yield a corresponding decrease in the time scales. In the same way, a faster system time evolution could be obtained by, e.g., an increase of either x_A or x_B or a decrease in x_a (see Eqs. (7), (8) and (9) which define the transition rates).

We have shown the numerical results for a energy landscape with $E_A = (E_B - E_a)$. No qualitative change in the function $q(f, r)$, with respect to that shown in Figure 3, has to be expected by changing slightly the value of one of the two barriers, while keeping the other one unchanged. Obviously, if one barrier becomes much larger than the other, the behaviour of the single barrier case is recovered.

With our choice for the model parameters, we have implicitly assumed that the inner barrier is the one which survives longer. Let t_0 be the time at which the inner barrier disappears, defined as $t_0 = E_A/(rx_A)$, and let t_{max} be the maximum time for the integration of the evolution equations (10) and (11). Thus, with our parameter choice, we took $t_{max} < t_0$. This corresponds to stop the kinetic process before the last barrier disappears: in the absence of barriers the Kramer formalism makes no longer sense. We now want to discuss shortly the case where it is the outer barrier to survive longer, i.e. $t_a > t_0$ (and $t_a > t_{max} > t_0$). This can be obtained, e.g., by taking $(E_B - E_a)$ moderately greater than E_A . In this case, a slightly modified version of equations (10) and (11) has to be considered to take into account the disappearing of the barrier (x_A, E_A) at $t = t_0$. The outcome for $q(f, r)$ is similar to that shown in Figure 3, but the slope at large force is $x_B - x_a$ rather than x_A . Still we believe that the choice $E_A \simeq (E_B - E_a)$, and $x_A \simeq (x_B - x_a)$ is the most

reasonable at this level of knowledge of the RNA tertiary structures, since each tertiary contact originates from the same physical mechanisms.

4 Discussion

The results shown in Figures 3 and 4 indicate that measuring $q(f)$ at different values of r sheds light on different parts of the energy landscape. Direct information on the outermost barrier can only be obtained by measuring $q(r, f)$ for very small values of r , while for high values of f , one obtains information on the innermost barrier. The model here presented also provides a method to obtain an experimental estimate of the intermediate energy minimum. The measurement of such quantity is highly desirable, since it determines the stability of the intermediate state with respect to molecule pulling.

In the moderate force regime, and for $r \geq 1$ pN/s, the quantity $q(f, r)$ does not give direct information on a single energy barrier, but rather indicates that in this force range there is a strong cooperativity in the unbinding process between the two kinetic barriers, that can be regarded as a single one.

In conclusion, we have proposed a simple model for the unfolding of RNA molecules with tertiary structure under dynamic loading, where two distinct kinetic barriers with similar height, hinder the system from opening. This choice leads the fraction of folded molecule to depend on the pulling rate, and therefore accounts for experimental results where apparently such a dependence has been observed [3]. In order for our model to be checked, the mechanical openings of a RNA molecule with tertiary contacts has to be performed with a wide range of pulling rate. The existence of a unique slope of $q(f)$ vs. f in the intermediate force range, and a scaling law for the zero-force transition rate, as the one we find out here, would unambiguously indicate that the single barrier picture is inadequate to describe the kinetic process corresponding to the mechanical breaking of an RNA tertiary structure. Our results suggest that the predominance of one of the two single kinetic barriers on the unfolding process, cannot be inferred by measuring the fraction of unfolded molecules in a relatively large range of force. On the contrary, the observation of this quantity over such an intermediate force range, suggests the existence of a complex kinetic barrier, which has a non trivial and unexpected connection with the single barriers. According to our model, in an unfolding experiment, a direct insight into the details of the single barriers can only be obtained either using a relatively small value of the pulling rate, or analyzing the unfolding kinetics in the large force regime. However the first possibility is limited by the need to keep the effect of apparatus drift under control.

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