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Single-molecule experiments in biophysics: Exploring the thermal behavior of nonequilibrium small systems

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Abstract. Biomolecules carry out very specialized tasks inside the cell where energies involved are few tens of $k_{\mathrm{B}}T$, small enough for thermal fluctuations to be relevant in many biomolecular processes. In this paper I discuss a few concepts and present some experimental results that show how the study of fluctuation theorems applied to biomolecules contributes to our understanding of the nonequilibrium thermal behavior of small systems.

Keywords. Biophysics; single-molecule experiments; fluctuation theorems.

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1. Biomolecules, molecular demons and statistical physics

Biophysics is a relatively young discipline that is becoming steadily popular among statistical physicists [1]. Although there are several reasons behind this general upsurge of interest, a very attractive aspect of biophysics is its strong interdisciplinary character. In recent years biophysics is facing the dawn of an unprecedented fusion of various knowledges coming from different traditional scientific areas from physics to chemistry, biology and computer science. At the root of such melting pot there is the discovery of the molecular structure of the gene by Crick and Watson in 1953. This has established the basis for a new 'solid state' science in biology, a bit akin to the role played in modern solid state and condensed matter physics by the discovery of the atom one century ago.

The current knowledge about the cell shows it as a very complex organism made out of several parts that carry out different specialized tasks organized into a modular structure, a bit like a farm or factory where different sections or departments are in charge of performing different tasks. This modular organization is extremely complex as it consists of different levels intertwined in a big fuss yet to be understood. The result of all these interactions is a web of informational flow where actions at one level trigger responses in another, cell differentiation being a prominent

example. Among these levels of complexity molecular biophysics is a discipline that aims to investigate the structure and function of biological matter from the physicochemical properties of molecules. Within this level it is nowadays possible, thanks to the development of nanotechnologies, to experimentally manipulate individual molecules while they carry out specialized molecular functions. In single-molecule experiments the information that can be gathered is fundamentally kinetic as molecules can be individually followed in time during a process which often occurs out of equilibrium. The merger of this knowledge with the static information gained from structural biology studies provides a promising framework to elucidate the function of many biomolecules.

One of the most crucial property of biomolecules (such as nucleic acid and proteins) is their capability to function as molecular machines or Maxwell demons that perform specialized molecular tasks under nonequilibrium conditions [2]. Often the innermost working of such machines is poorly understood, however one common aspect is their non-deterministic behavior (contrary to the working of macroscopic machines). The surrounding water is the thermal bath. This acts as a thermal bath by allowing biomolecules to exchange energy with the molecules of the solvent through the breakage of weak molecular bonds. The amount of energy typically exchanged during the excursions of the molecular machine correspond to those delivered in collisions between the molecules of the solvent and the atoms in the biomolecule that trigger the relevant conformational changes. Considering that each molecule of the solvent carries $\sim 1k_{\rm B}T$ ($k_{\rm B}$ being the Boltzmann constant and T the temperature of the bath) then the energies exchanged amount to a few times $k_{\rm B}T$. This number is roughly equal to the number of weak bonds that must be broken to trigger the conformational change. For example, during the replication of DNA, the replication fork advances one base pair (about 1/3 of a nanometer) every time the DNA polymerase (a molecular machine) adds one nucleotide to the newly synthesized DNA strands. The forces that keep the polymerase moving during this nonequilibrium process are generated from ATP consumption during the hydrolysis cycle. Often molecular machines do not act alone but rather a multiplex of several proteins are involved in the most basic tasks carried out inside the cell. For example, in the aforementioned case of DNA replication, there is a forerunner of the DNA polymerase, known as the helicase. The task of this enzyme is the progressive unwinding of the double helix as the polymerase advances (see figure 1). Sustained by ATP consumption the helicase exerts mechanical work upon the DNA, a by-product of the mechanical torque exerted on the helix and the angle of unwinding required for the exposure to the polymerase of the successively unwounded base pairs.

Despite the enormous complexity of the whole replication process, it is interesting to ask how each of these individual motors work (e.g. in the case of the helicase) and what is the amount of energy consumed at periodic time intervals. Surely enough this quantity will strongly fluctuate as the behavior of these machines is stochastic and ATP consumption is not deterministic. Although energy consumption is a rather tricky quantity, mechanical work turns out to be experimentally measurable as single-molecule techniques allow us to measure forces (or torques) and distances (or angles). Mechanical work is also a stochastic quantity and so we may ask what is the distribution of the work done by the helicase and measured along many time intervals of a given duration. For macroscopic machines, if stochasticity was

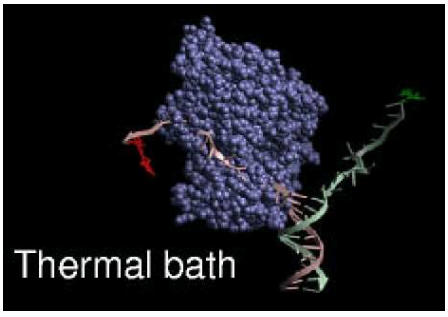

Figure 1. The helicase is an enzyme involved in the unwinding of the DNA helix that paves the way for the replication process carried out by the DNA polymerase. Its behavior is thought to be stochastic and intermittent.

experimentally observable, we might expect a work distribution dominated by an extremely narrow Gaussian component as predicted by the law of large numbers. However, for small machines the distribution might be strikingly different as their inner workings is a by-product of progressive evolution after millions of years. Quite probably, the distribution will be strongly non-Gaussian and intermittent [3], an economy saving strategy for information transfer [4]. This means that most of the time the helicase does nothing while jiggling at a fast frequency around its local equilibrium position. However, from time to time and at a much lower frequency, the helicase hydrolyzes one ATP molecule and makes a conformation change that triggers the unwinding of an additional base pair.

The discipline that investigates the thermal behavior of small systems under various nonequilibrium conditions is called thermodynamics of small systems. It addresses the question about the statistical description of energy exchange processes in small nonequilibrium systems embedded in thermal environments where the relevant exchanged energies are few times (N) $k_{\mathrm{B}}T$. So relative deviations (of order $1/\sqrt{N}$) are not negligible over time-scales relevant to biomolecular processes. The plan of the paper is as follows: In §2, I describe a few concepts that are central in a thermodynamic description of nonequilibrium small systems. In §3, I briefly discuss the usefulness of fluctuation theorems to describe energy exchanged fluctuations in nonequilibrium processes. Section 4 describes single-molecule experiments as a promising route to investigate such fluctuations. Finally I show some recent results regarding work fluctuations in the mechanical unfolding of RNA molecules (§5).

2. Small systems: Heat, work and fluctuations

A central notion in thermodynamics of small systems is the concept of control parameter [5]. This is akin to the concept of external variable used to define ensembles in statistical mechanics. The main difference between a thermodynamic description of macroscopic and small systems is that, in the former, fluctuations are not essential to characterize thermodynamic transformations. When fluctuations are included it is only by considering small deviations (typically Gaussian distributed)

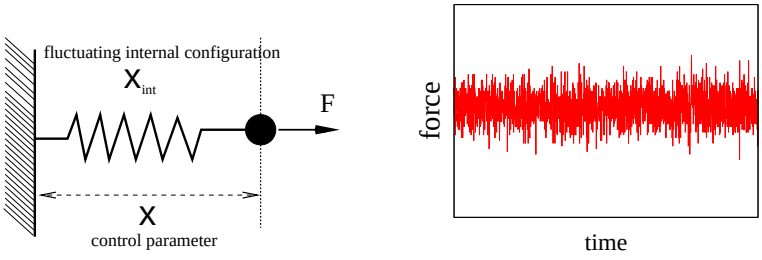

Figure 2. (Left panel) Schematic picture of a small spring in contact with a bead held at distance x to the wall. The force exerted on the bead fluctuates with time (right panel), the spectrum of force fluctuations being Gaussian for small deformations of the spring.

around the average macroscopic value. Instead, large non-Gaussian deviations are irrelevant as they are extremely unlikely. For small systems a description in terms of average values does not suffice, in particular when describing nonequilibrium thermal processes where rare and large deviations often occur. When embedded in a thermal environment every observable of a small equilibrated system strongly fluctuates. In order to define an equilibrium state it is then convenient to specify the control parameter. This is a non-fluctuating parameter that, once fixed, determines the fluctuating spectrum of the other variables. Unlike macroscopic thermodynamics, different equilibrium states can exist for small systems, depending on which parameter is controlled externally.

In order to understand the meaning of the control parameter better, let us think of the following *Gedanken* experiment. In figure 2 we show a small bead connected to the extreme of an overdamped spring whose other extreme is held fixed to a wall. The whole system is embedded in a thermal bath kept at a given temperature and pressure. However, unlike macroscopic systems, we will assume that the spring is small and made out of few hundreds of atoms (e.g. this could be a polymer made out of few hundreds of monomers). The configuration of the system is then specified by an internal set of variables $\{x_i\}$ specifying the positions of all atoms of the spring as well as the bead. In equilibrium, and in the absence of any other interaction with the external world, the extension of the spring will fluctuate around a reference value that we take equal to zero. If we want to pull the spring there are different ways to do that. One way would be to pull the bead by moving the distance $x(t)$ in a controlled way. In this case x is the control parameter and the internal configuration of the spring and the force acting on the bead will fluctuate (figure 2). For arbitrary deformations (described by x) the average force acting on the bead will satisfy $\langle F\rangle = f(x)$, f being a given function with $f'(0) = k$, equal to the stiffness of the spring. On the other hand, we could pull the spring by controlling the force (e.g. by applying an external magnetic field to a magnetized bead). In this case the force would be fixed but the distance x would fluctuate and satisfy $F = g(\langle x\rangle)$ with g another function. In general, $f \neq g$ so the equilibrium state is different in both protocols (distance- or force-controlled). Only for macroscopic systems $f = g$ and both set-ups are equivalent.

Let a system be described by an internal configuration $\{x_i\}$ and a control parameter that we will denote as x (in general there can be a finite number of control

parameters). Let $U(\{x_i\}, x)$ describe the internal energy of the system. Upon variation of x the energy will change,

$$\mathrm{d}U(\{x_i\}, x) = \sum_i \left(\frac{\partial U}{\partial x_i}\right)_x \mathrm{d}x_i + \left(\frac{\partial U}{\partial x}\right)_{\{x_i\}} \mathrm{d}x = \mathrm{d}Q + \mathrm{d}W \tag{1}$$

which is the content of the first law of thermodynamics (i.e. energy conservation). Now let us consider a process where the spring, initially in thermal equilibrium at $x = 0$, is pulled by changing the control parameter according to a perturbation protocol $x(t)$ in a process that lasts for a time t_f ($x(t_f) = x_f$). If the speed $\dot{x}$ is much larger than the relaxation frequency of the system $\omega = k/\gamma$ (γ being the friction coefficient of the bead), then the system will be driven out-of-equilibrium during the process. The total work done on the system is given by

$$W = \int_0^{x_f} F(\{x_i\}, x)\mathrm{d}x, \tag{2}$$

where $F(\{x_i\}, x)$ is the fluctuating force acting upon the bead,

$$F(\{x_i\}, x) = \left(\frac{\partial U}{\partial x}\right)_{\{x_i\}}. \tag{3}$$

If we repeat this nonequilibrium experiment many times, always starting from the same equilibrated state at $x = 0$ and following the same protocol $x(t)$, the system will follow different trajectories (i.e. the time evolution of $\{x_i\}$ and therefore (3) will change from experiment to experiment). Consequently, the total work (2) will also fluctuate from experiment to experiment. A quantity that characterizes the nonequilibrium process is the probability distribution $P(W)$ of work values obtained along different trajectories. The discussion of some of the mathematical properties of this distribution is the main subject of concern in this paper, the

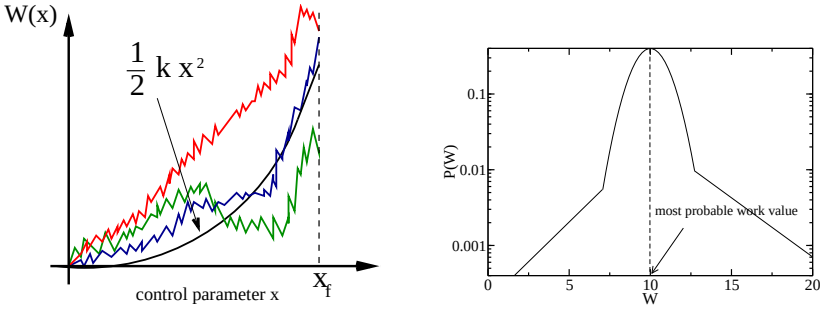

Figure 3. Fluctuations in the work exerted upon a small spring immersed in a thermal bath (left panel). The continuous black line is the average work for small deformations (k is the stiffness of the spring). In general, the probability distribution of the work exerted upon the system along many repeated experiments (right panel) will have two sectors characteristic of intermittent behavior: a large Gaussian component describing small and frequent fluctuations and exponential tails describing large and rare deviations. Only for linear systems (i.e. for small deformations) fully Gaussian behavior is recovered [3].

quantity that characterizes the small system during the nonequilibrium process and a fingerprint of its nonequilibrium behavior (figure 3).

3. Free-energy recovery from nonequilibrium experiments

In a nonequilibrium process the second law of thermodynamics [6] establishes that the work averaged over all trajectories $\langle W\rangle = \int WP(W)\mathrm{d}W$ is larger than the reversible work (equal to the free-energy difference ΔG between the equilibrium states defined at $x = x_f$ and $x = 0$). If we define $W_{\rm dis} = W - \Delta G$ as the dissipated work along a given trajectory, then the content of the second law can be written as

$$\langle W\rangle \geq \Delta G \;\rightarrow\; \langle W_{\rm dis}\rangle \geq 0. \tag{4}$$

The equality occurs only when the perturbation process is carried out infinitely slowly in a quasi-static process where $\dot{x} \to 0$. In such a process the system is given enough time to relax to equilibrium at each value of the control parameter, and therefore $W = \Delta G$ and $P(W) = \delta(W - \Delta G)$ or $P(W_{\rm dis}) = \delta(W_{\rm dis})$ (this is true for stochastic but not for deterministic dynamics). Nonequilibrium processes are characterized by hysteresis phenomena, the average work performed upon the system differs between a given process and its time-reversed one. Fluctuation theorems assert relations between the entropy production along a given process (usually termed as forward) and their reversed one [5,7]. In the aforementioned example of the spring, let $x_{\rm F}(t)$ stand for the forward protocol that pulls the spring from $x = 0$ to $x_{\rm F}(t_f) = x_f$. The time reversed protocol is then defined by $x_{\rm R}(t) = x_{\rm F}(t_f - t)$. Under the assumptions that the system is microscopically reversible (detailed balance) and that the system starts at equilibrium at $x = 0$ in the forward process and at $x = x_f$ in the reverse process, the following result has been derived by Crooks [8]:

$$\frac{P_{\rm F}(W)}{P_{\rm R}(-W)} = \exp\Big(\frac{W - \Delta G}{k_{\rm B}T}\Big), \tag{5}$$

where $P_{\rm F}(W), P_{\rm R}(-W)$ are the work distributions along the forward and reverse processes respectively (the minus sign in the argument of the reverse work distribution arises from the corresponding reverse sign of $\mathrm{d}x$ in (2)). Equation (5) has the form of a fluctuation theorem (FT) and quantifies the amount of hysteresis for arbitrary nonequilibrium protocols. The quasi-static process is a particular case of (5) where $W = \Delta G$ and there is no hysteresis between the forward and the reverse paths.

A straightforward consequence of the Crooks FT is the Jarzynski equality (JE) [9]. By rewriting (5) and integrating out the distribution $P_{\rm R}(-W)$ over W it is possible to derive the following expression:

$$\Big\langle \exp\Big(-\frac{W}{k_{\rm B}T}\Big)\Big\rangle_{\rm F} = \exp\Big(-\frac{\Delta G}{k_{\rm B}T}\Big) \quad \text{or} \quad \Big\langle \exp\Big(-\frac{W_{\rm dis}}{k_{\rm B}T}\Big)\Big\rangle_{\rm F} = 1, \tag{6}$$

where the average $\langle\cdots\rangle_{\rm F}$ is taken over all possible work values along the forward process. A consequence of JE is the second law $\langle W_{\rm dis}\rangle_{\rm F} \geq 0$ that can be derived

by applying Jensen's inequality ($\langle \exp(x) \rangle \geq \exp(\langle x \rangle)$). The content of JE is that, albeit the average dissipated work is positive, tails in the work distribution that extend to the region $W_{\rm dis} < 0$ must exist for the equality to be satisfied. Trajectories contributing to these tails are often called transient violations of the second law because they violate the inequality (4) for a single trajectory. It has to be stressed, however, that no violation of the second law occurs as the content of the inequality only concerns the average value of the work rather than the value of the work of individual trajectories. The validity and consistency of the Crooks FT and the JE have been recently put under scrutiny [10]. Yet, the experimental validity of such a theorem has been recently tested in RNA pulling experiments [11,12] in what represents an important step in our understanding of fluctuations in small systems.

Additional interest in the Crooks FT and the JE stems from the fact that these results can be used to recover equilibrium free-energy differences from nonequilibrium experiments. This has applications in numerical simulations of molecular reactions which often cannot be investigated using equilibrium methods [13], or single-molecule experiments where free-energy measurements cannot be carried out reversibly [5,12]. In fact, rewriting (6) as

$$\Delta G = -k_{\rm B}T \log \left(\left\langle \exp\left(-\frac{W}{k_{\rm B}T} \right) \right\rangle \right), \tag{7}$$

shows that by exponentially averaging the nonequilibrium work it is possible to recover the value of the reversible work (equal to the free-energy difference). As always there is no free lunch, and the main disadvantage of (7) lies on the fact that the average $\langle \cdots \rangle$ must be taken over an infinite number of nonequilibrium trajectories. The number of available trajectories is always finite, and therefore the risk exists that some of the trajectories which mostly contribute to the exponential average are not picked out. Indeed, this is precisely what happens, as the most improbable trajectories that populate the negative tail of the work distribution are the ones that mostly contribute to (7). How many nonequilibrium experiments are needed in order to recover the free-energy within a given accuracy is one of the most useful questions one would like to answer. It can be shown that the exponential average in (7) is a biased quantity [14,15], and such number of experiments increases exponentially fast with the average value of the dissipated work [16]. Nevertheless, the precise value of the prefactor and the factor in the exponential depend in a complicated way on the left tails of the work distribution.

The Crooks FT can also be used for free-energy recovery by applying the so-called crossing methods [12]. Indeed, from (5) we infer that for $W = \Delta G$ both distributions (forward and reverse) cross each other allowing to extract the value of ΔG,

$$P_{\rm F}(W) = P_{\rm R}(-W) \rightarrow W = \Delta G. \tag{8}$$

Further improvement of the crossing method uses information from both distributions along the whole work-axis rather than only local behavior around $W = \Delta G$. To this end we consider the two functions

$$\Omega(z) = \frac{N_{\rm R}(-W > z)}{N_{\rm F}(W > z)}; \qquad \Phi(x) = \left\langle \exp\left(-\frac{(W - z)}{k_{\rm B}T} \right) \right\rangle_{{\rm F}, W > z}, \tag{9}$$

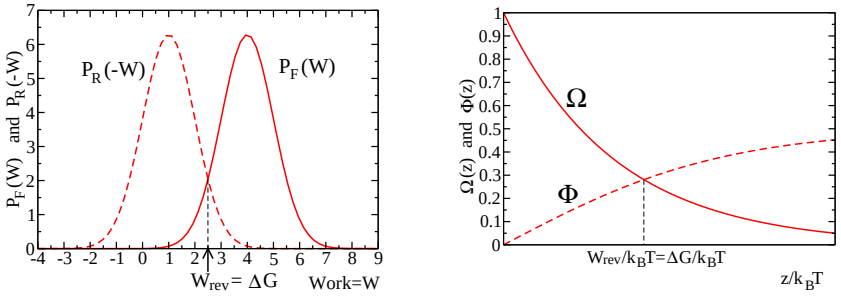

Figure 4. Crossing methods to determine ΔG from nonequilibrium work measurements.

where $N_{\rm F}(W > z), N_{\rm R}(-W > z)$ indicate the fraction of trajectories with work values larger than z along the unfolding and refolding paths respectively. The average $\langle \cdots \rangle_{{\rm F},W>z}$ is restricted over the set of trajectories along the forward process with work larger than z. These functions satisfy the following properties: (a) $\Omega(z)$ is a monotonically decreasing function starting at 1 for $z \ll \Delta G$ and decaying to zero for $z \gg \Delta G$; (b) $\Phi(z)$ is a monotonically increasing function starting at 0 for $z \ll \Delta G$ which saturates for $z \gg \Delta G$; (c) both functions cross each other at $z = \Delta G$. The two methods are exemplified in figure 4 for the case of Gaussian work distributions (this case corresponds to a bead confined in an optical trap which is dragged through water [17,18]).

4. Single-molecule force microscopy

As we have seen in the preceding sections, mechanical work plays a central role in a thermodynamic description of small systems as there are specific relations that quantify their probability distributions. From the experimental point of view, force microscopies provide a tool to manipulate individual biomolecules by applying mechanical force at their ends. In this way it is possible to exert mechanical work upon these molecules and, by repeated pullings, to determine experimentally the work probability distribution in a given nonequilibrium process.

There are several kinds of force microscopies, the most well-known are atomic force microscopy, magnetic and optical tweezers. The latter are particularly suitable as the range of forces they can exert are in the range 1–100 pN relevant to many weak interactions participating in biomolecular processes. Laser tweezers (see figure 5) use the principle of conservation of light momentum to exert forces on small micron-sized polystyrene beads due to light deflection of the beam as it changes medium between water and the bead [19]. In this way a bead is trapped into the focus of the laser, the configuration of minimal energy. When the bead deviates from the focus a restoring force acts upon the bead, the principle being the same by which a dielectric substance inside a capacitor is drawn inwards by the action of the electric field. To a very good approximation the trap potential is harmonic, therefore the restoring force acting on the bead is linear with the deviation of the bead from the center of the trap. Calibration of the optical

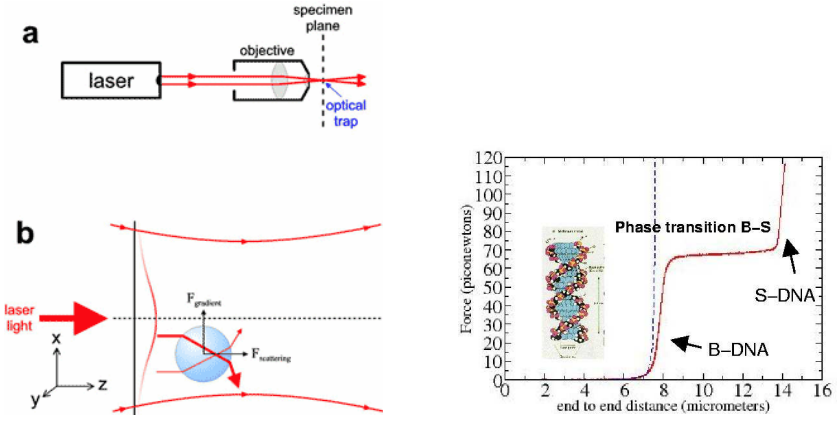

Figure 5. Left panel: Physical principles of the single-beam laser tweezers. The set-up is made up of a laser and and an objective (**a**) which is focused on a spot. A micron-sized bead is pulled towards the region of maximum light intensity (**b**). Right panel: Force-extension curve (FEC) in a 24 kbp fragment of λ-DNA (torsionally unconstrained) showing the overstretching transition at 65 pN. The dashed line is the worm-like-chain prediction which does not include the elastic rigidity of the backbone.

trap allows us to determine the force acting on the bead by reading the deviation of the bead from the center of the trap, inasmuch as the position of the needle in a manometer indicates the value of pressure of a fluid or a gas. A typical value of the trap stiffness is 0.1 pN/nm. In general, it is more convenient to use dual-beam optical tweezers which do not need continuous calibration as the force can be determined by the total amount of light collected by two photodetectors sitting at the opposite sides of the beams (see figure 6). Experiments use micron-sized glass chambers filled with water and two beads. Molecules are chemically labeled at their ends and polystyrene beads are chemically coated to stick to the ends of the labeled molecule. In this way a tether can be made between the two beads. One bead is held fixed by air suction on the tip of a glass micro-pipette, the other is trapped in the focus of the laser and used to measure the force applied to the molecule. A frame-grabber and a light-lever then measure the extension of the molecule with a precision down to the nanometer range.

The outcome of these experiments are the so-called force-extension curves (FECs) where the force acting on the molecule is represented as a function of the end-to-end distance between the two beads. In this way it has been possible to experimentally check whether DNA behaves as some polymer theories predict [20]. At small forces (below 1 pN) the polymer behaves like a Hookean entropic spring as described by the freely jointed chain model [21]. At larger forces deviations occur and the FEC is well-described by the worm-like chain model. Above 5 pN enthalpic contributions due to the finite rigidity of the sugar-phosphate backbone start to be important. Finally, at 65 pN a force plateau is observed characteristic of a transition between the B-DNA form and a stretched new form of DNA (termed as S-DNA) [22,23] (see figure 5). FECs provide insight into the inner-workings of

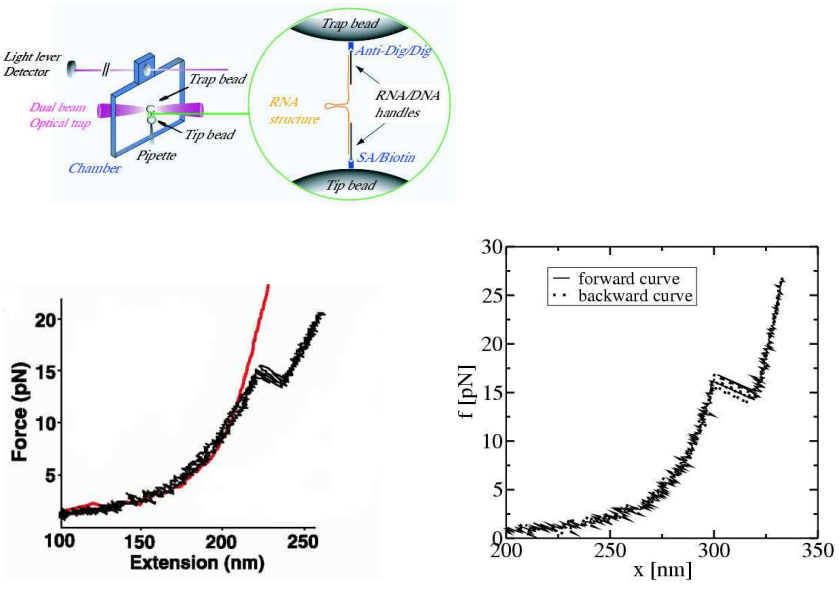

Figure 6. Upper panel: Experimental set-up for dual laser tweezers in RNA pulling experiments. Lower panel: FEC for a small RNA hairpin showing the rip in the force indicating the unfolding of the molecule. Experiments have been done in [24] (left panel) and later compared with theoretical models [26] (right panel).

biomolecules. The unfolding of RNA molecules or proteins under the action of mechanical force is a case of interest. Under physiological conditions these molecules are in a folded or native, functionally active, conformation. Upon heating or chemical treatment they denature and degrade into an extended, functionally inactive, conformation. The thermodynamic stability of the native state is determined by the free-energy difference ΔG between the two conformations. Upon the action of mechanical force RNA hairpins denaturate as revealed by the presence of a rip in the FEC [24] (see figure 6). These experiments allow us to obtain estimates of ΔG by measuring the mechanical work exerted upon the molecule across the transition. In addition, hopping effects between the folded and the unfolded conformations also yield valuable information about the kinetics of unfolding in the presence of force [25], a process thought to be relevant during the synthesis of proteins (in the translation–elongation process) in the ribosome. Because of the short extension of the unfolded hairpins (few tens of nanometers) as compared to the size of the beads, the experimental set-up in RNA pulling experiments is a bit more elaborate than when pulling DNA (see figure 6). To harness the RNA molecule two RNA/DNA hybrid handles (typically a few hundred nanometers long) are attached to its ends. These handles act as transducers of the force and have direct influence on the unfolding kinetics of the RNA molecule. A proper inclusion of all the elements in the experimental set-up (such as the bead in the trap and the handles) and the correct identification of the control parameter are required to extract information about the RNA molecule (e.g. the value of ΔG or the kinetic rates) [26].

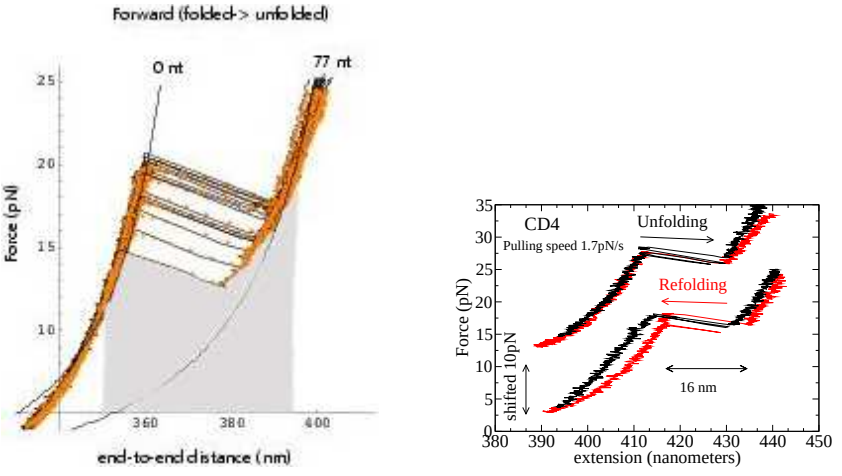

Figure 7. Left panel: Pulling curves in the S15 three-way junction exhibit the strong work fluctuations observed (measured by the gray area under the FEC). Right panel: Drift effects in the quasi-reversible unfolding of a short RNA hairpin. For the sake of clarity the second pulling cycle has been shifted 10 pN upwards.

Picking up the threads of our main theme, RNA molecules are specially suitable to measure work fluctuations. The reason lies in their modular structure where large RNA molecules are made out of different motifs or units that unfold sequentially upon pulling [27]. The value of ΔG for each structural motif is typically a few tens of $k_{\mathrm{B}}T$ and each one usually dissipates a few $k_{\mathrm{B}}T$ when unfolded irreversibly. This quantity is small enough for work fluctuations, as determined by the value of the force at which the rip occurs, to be experimentally observable. In figure 7 we show work fluctuations as measured from the area below the FEC [27a].

5. Predicting unfolding free-energies of RNA motifs from irreversible measurements of mechanical work

As we already said, pulling experiments allow us to extract information about the unfolding chemical reaction of both thermodynamic character (the value of ΔG) and kinetic character (the reaction rate). Here we want to discuss more about how to extract the value of ΔG in RNA molecules. Traditionally, ΔG is extracted from calorimetry experiments by integrating the specific heat as a function of the temperature across the melting transition. However, in contrast to proteins, some RNA molecules melt at temperatures above the boiling point of water, precluding the use of calorimetric measurements. It is therefore convenient to find new routes to extract the free-energy of the folded state for such molecules. As we have said, the measure of the reversible work across the transition in pulling experiments would be a direct measurement of ΔG. Unfortunately, in most interesting cases (e.g. RNA molecules with tertiary interactions induced in the presence of Mg^{2+} ions), the unfolding reaction is so slow that it cannot be carried out reversibly at

the available lowest pulling speeds (largely limited by the presence of strong drift effects in the laser tweezers machine, see figure 7). Therefore, other strategies must be envisaged such as the use of the Crooks FT discussed in §3 [11].

6. Conclusions

The use of single-molecule techniques allows us to investigate the nonequilibrium behavior of biomolecules. Such study reveals the presence of strong thermal fluctuations due to the smallness of the typical energies associated to the physical interactions in biomolecules (of the order of few tens of $k_{\mathrm{B}}T$). These energies are small enough for large deviations respect to the average value be experimentally observable and important in the time-scales relevant to many biomolecular processes. Weak molecular bonds (Van der Waals non-specific binding, hydrogen bonds or hydrophobic interactions) are the leading interactions responsible for many such processes. They are behind molecular recognition and drive the transfer of information between biomolecules. It is not by accident that weak interactions, which induce strong energy fluctuations, dominate the inner workings of life processes at the molecular level. It is quite likely that the large and intermittent fluctuations characteristic of biomolecules play an important role in the way molecular evolution has reached such exquisite degree of complexity. This facilitates that large groups of weakly interacting biomolecules cooperate and carry out very specialized tasks.

Establishing the nature of work and heat fluctuations in biomolecules seems therefore relevant to understand the principles underlying the organization of biological matter at the nanoscale. Statistical physics provides concepts and tools to address such questions and fluctuation theorems appear as a good playground to elucidate many aspects concerning thermal fluctuations in nonequilibrium small systems. Here we have reviewed a few ideas and experiments in this exciting field of research which combines knowledge from different areas of expertise, ranging from physics to chemistry and biology. Sure enough we will see exciting scientific developments in the future that will help us to better understand the nonequilibrium thermal behavior of small systems.

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