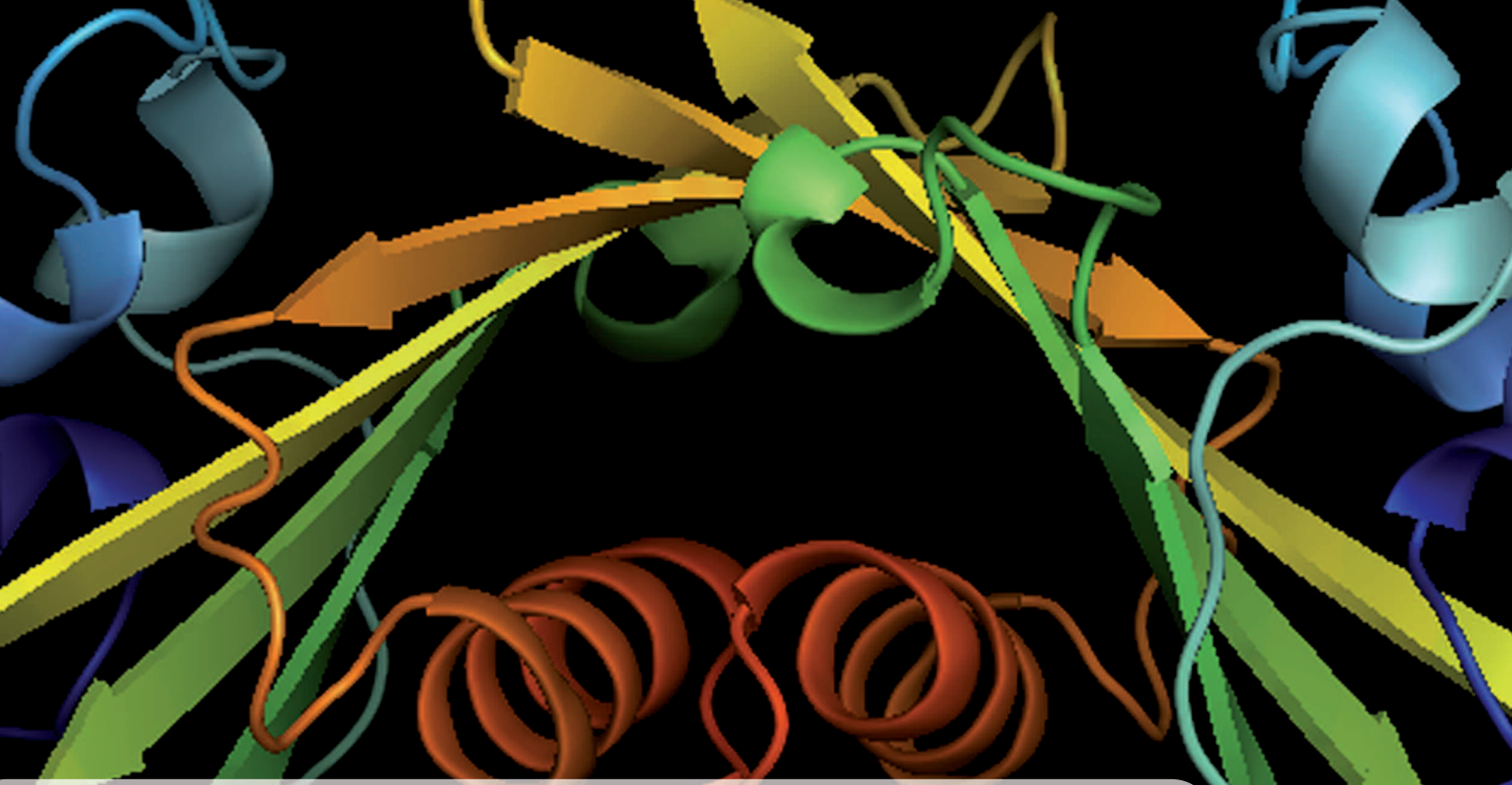

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FLUCTUATION THEOREMS IN SMALL SYSTEMS:

EXTENDING THERMODYNAMICS TO THE NANOSCALE

Recent developments in micro and nanotechnologies enable the manipulation of single molecules one at a time. Such experiments make it possible to resolve energy processes with unprecedented detail at the level of 1 k_BT, the typical energy scale of Brownian fluctuations. Fluctuation theorems establish relations governing energy exchange processes at this level and provide a new methodology to obtain equilibrium information from non-equilibrium experiments.

Biophysics is the branch of science that aims to understand biological processes using concepts and tools from mathematics, physics and chemistry. Molecular biophysics focuses the attention on fundamental processes occurring at the molecular level, like the action of molecular motors [1], the folding of proteins [2], the physical chemistry of nucleic acids [3] or molecular transport through ionic channels [4], among many others. Traditionally, experiments have been done on samples containing a quantity of molecules of the order of the Avogadro number, $\approx 10^{23}$. For example, in calorimetry or nuclear magnetic resonance samples contain trillions of molecules in solution. Thus, measurements are an average over individual behaviours and thermodynamics is the branch of science needed to describe the equilibrium properties observed. Fluctuations often do not play any relevant role as they are too small: their relative magnitude scales as $1/\sqrt{N}$, N being the total number of molecules.

Thanks to recent developments in technology, scientists can investigate the properties of single molecules at high spatial and temporal resolution. Optical tweezers (Box 1), atomic force microscopes, or magnetic tweezers allow us to grab and manipulate one molecule in each experiment and to obtain precious information often inaccessible to bulk methods [5]. Single molecule experiments (SME) make it possible to probe small energies comparable to the energy unit of thermal noise, k_BT (k_B being the Boltzmann constant and T the ambient temperature). To emphasize the smallness of the energy scale, these systems are usually referred to as "small systems". In such conditions, thermal fluctuations

▲ Characteristic 3D structure of the Ivy protein (Inhibitor of vertebrate lysosyme) used by numerous bacteria to be protected from lysosyme. ©CNRS Photothèque C. Abergel lab.: UPR2589 - IGS - marseille

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are relevant and deviations from the average behaviour are observable. To be reliable, SME must be repeated several times in order to identify a reproducible pattern in the measurements. Thermodynamics is still applicable, although big relative energy fluctuations are the rule: in such a noisy environment, fluctuation theorems provide a novel conceptual framework useful to characterize a molecule from non-equilibrium SME.

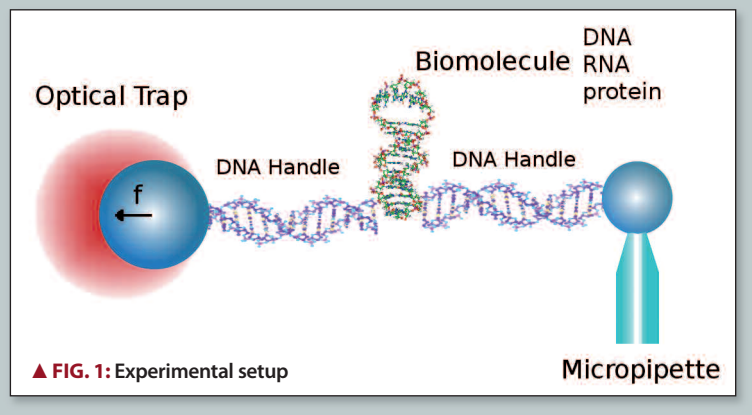
Unzipping experiments

Biomolecules (*e.g.* RNA, DNA or proteins) stretched under force are excellent models to explore the non-equilibrium physics of small systems. The great versatility of DNA synthesis together with its high molecular stability makes it a suitable system to work with. DNA hairpins (fig. 2) are among the simplest DNA structures important in many biological processes. They are formed of a stable DNA double helix (called 'stem') ended by a loop. The number of base pairs in the double helix and the size of the loop are parameters that we can control. This allows us to design DNA molecules with specific thermodynamic and kinetic properties.

Single molecule experiments (SME) make it possible to **probe small energies** comparable to the energy unit of **thermal noise, k_BT**

Optical Tweezers and Single Molecule Experiments

Optical tweezers allow us to manipulate tiny objects with sub-nanometer resolution and to exert mechanical forces in the picoNewton range. By optically trapping micron-sized beads it is possible to grab and pull biomolecules that are a thousand times smaller than the beads [9].
Molecular biology tools are employed to insert a molecule between molecular handles. This complex is then tethered between two micron-sized polystyrene or silica beads.



▲ **FIG. 1:** Experimental setup

One bead is captured in the optical trap, while the other is immobilized by air suction on the tip of a micropipette (see figure). The bead in the optical trap feels a force towards the center of the trap. This force is then transmitted to the molecular system in mechanical equilibrium. Handles act as force transducers and provide free space to avoid spurious interactions between the molecule under study and the beads.

In unzipping experiments [6], a DNA hairpin is converted into a single DNA strand by pulling apart the two chains of the double helix from the same end (see Box). By applying force, weak molecular interactions are disrupted and energies of a few k_BT (or kcal/mol) are probed. Unzipping occurs around 15 pN, which is the force required to overcome the hybridization forces that bind the two complementary strands of the double helix. When unzipping long DNA hairpins (a few hundreds of base pairs) at a constant speed a force-distance curve (FDC) with a sawtooth pattern is observed (fig. 3a) [7]. At each force rip some base pairs are released and the DNA hairpin becomes progressively opened, until it unfolds completely.
In short hairpins (a few tens of base pairs) a single rip is observed revealing a two-states behavior: at low forces the molecule is in its native state, while at high forces the unfolded and stretched state becomes thermodynamically stable (fig. 3b) [8]. The rupture process is thermally activated (stochastic), so the value of the force at which the hairpin unfolds changes at each unzipping experiment under the effect of thermal fluctuations (fig 3c). Once the molecule is in the unfolded state, we can recover the native structure by releasing the force applied to the system. The latter process is called zipping.

Fluctuation Theorems

Fluctuation Theorems (FT) establish fundamental relations about energy exchanges between a non-equilibrium system and its environment, and allow one to obtain equilibrium information from non-equilibrium experiments [10]. SME are an ideal laboratory to explore FT.
To better understand FT let us consider a small system, prepared in an arbitrary initial state and kept in contact with a thermal bath, that is submitted to an external time-dependent perturbation. Any perturbation can be described by a macroscopic coordinate of the system that is reproducibly changed from an initial to a final value following a specified protocol. The work W measured along the path or trajectory will vary from experiment to experiment because we cannot control the microscopic configurations explored by the system under the perturbation (*i.e.*, work is a path-dependent variable). As work is an extensive variable, these fluctuations are suppressed in experiments with a large number of particles. However, in SME work fluctuations are significant because of the small energies involved. For infinitely slow perturbations, the adiabatic hypothesis tells us that the process is reversible and the work is equal to the difference in free energy between the final and initial states, $W_{rev}=\Delta G$.
However, the faster we apply the perturbation, the more the mean work $\langle W \rangle$ increases (averaged over a large number of experiments undergoing the same protocol). Because the free energy remains the same, the average

dissipated work $\langle W_{dis}\rangle=\langle W\rangle-\Delta G$ also increases. The second law of thermodynamics asserts that the average dissipated work is always positive for irreversible transformations, and equal to zero only when the system evolves under reversible conditions, $\langle W\rangle \geq \Delta G \Leftrightarrow \langle W_{dis}\rangle \geq 0$.

It is crucial to bear in mind that thermodynamics apply for averages over an infinite number of trajectories generated under identical conditions. They are not applicable to single trajectories.

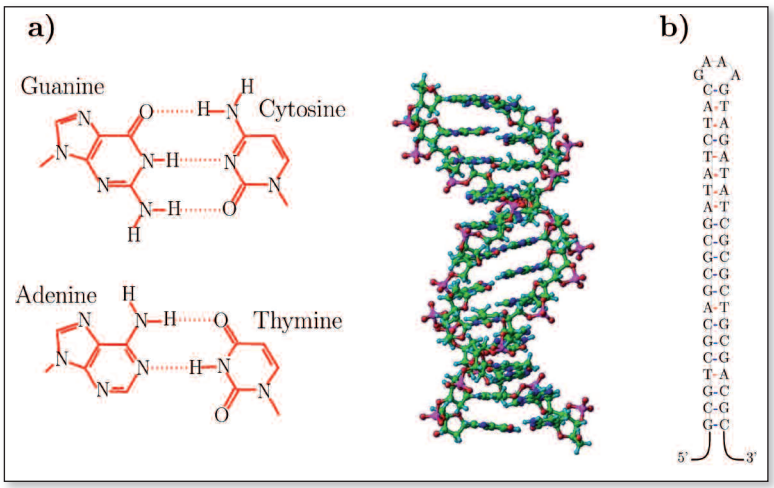
In 1997 C. Jarzynski proved a remarkable result about non-equilibrium physics [11]. Think of a system initially in thermal equilibrium with a bath at temperature T. Then a time-dependent perturbation is applied to the system and it evolves to a final state. If we assume that the microscopic dynamics of the system is Markovian then one can prove Jarzynski equality (JE):

$$\left\langle \exp\left(-\frac{W}{k_B T}\right)\right\rangle = \exp\left(-\frac{\Delta G}{k_B T}\right), \qquad (1)$$

where k_B is the Boltzmann constant. The average is taken over an infinite number of independent trajectories undergoing the same perturbation protocol. Using the JE one can recover the value of the reversible work, or the free energy difference, from measurements of the irreversible work. Moreover, it does not matter how far from equilibrium the system is driven during the experiment. Remarkably, the second law of thermodynamics can be inferred from the JE.

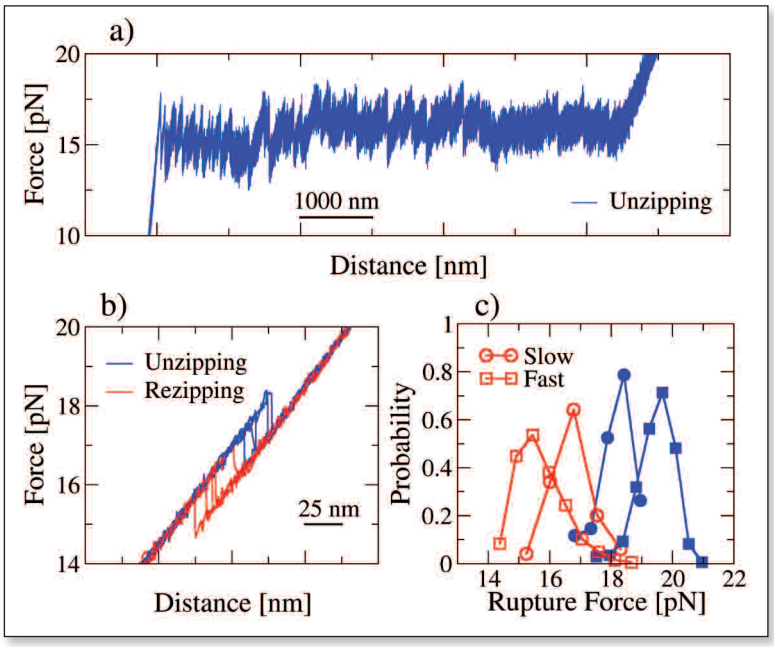
The JE implies the presence of trajectories where the dissipated work is negative, meaning that heat from the environment is absorbed by the system and converted into useful mechanical work. Despite appearances, it must be stressed that there is no violation of the second law whatsoever, because thermodynamics does not focus on single trajectories but on the average over an infinite number of them. The recovery of the reversible work from measurements of the irreversible work entails the realization of an infinite number of experiments. However, experimentalists only perform a limited number of measurements. The average of the exponential of the work becomes a finite sum of random exponentials, a strongly biased quantity displaying slow convergence to its limit. When applying the JE the most rare trajectories with smaller work values are unfairly weighed and the experimental free energy obtained is biased toward values larger than the true free energy.

Figure 4 shows results obtained with the DNA hairpin sketched in figure 2b. The pulling speed in experiments was set to 180 nm/s. The predicted free energy of formation of this hairpin at 25°C and 1M NaCl, obtained from calorimetric melting experiments, is 60.5 k_BT (35.85 kcal/mol). The free energy recovered by measuring the work (fig. 4a) and using the JE equals the free energy difference of the total system



▲ **FIG. 2: a)** DNA consists of two complementary backbones made of phosphate groups and sugars. DNA bases, guanine (G), cytosine (C), adenine (A) and thymine (T), are attached to each sugar, and its sequence encodes genetic information. The two strands form a double helix and are held together by the hydrogen bonds established between the different bases (A interacts with T and G with C). **b)** A DNA hairpin consists of a DNA double ended by a loop. The formation of the loop costs bending and twisting energy and involves a decrease in entropy. Yet, the large stability of the stem makes the whole structure thermodynamically stable. In the example, a DNA hairpin of 21 base pairs is sketched.

▼ **FIG. 3:** Examples of FDC obtained with optical tweezers. **a)** Unzipping experiment carried out at 25 nm/s (almost reversibly). The hairpin stem is 6838 base pairs long. **b)** Unzipping and zipping trajectories obtained for the hairpin shown in fig. 1b pulled at 180 nm/s. **c)** Histograms for the rupture forces obtained at different unzipping/zipping speeds: 60 (slow) and 180 (fast) nm/s.

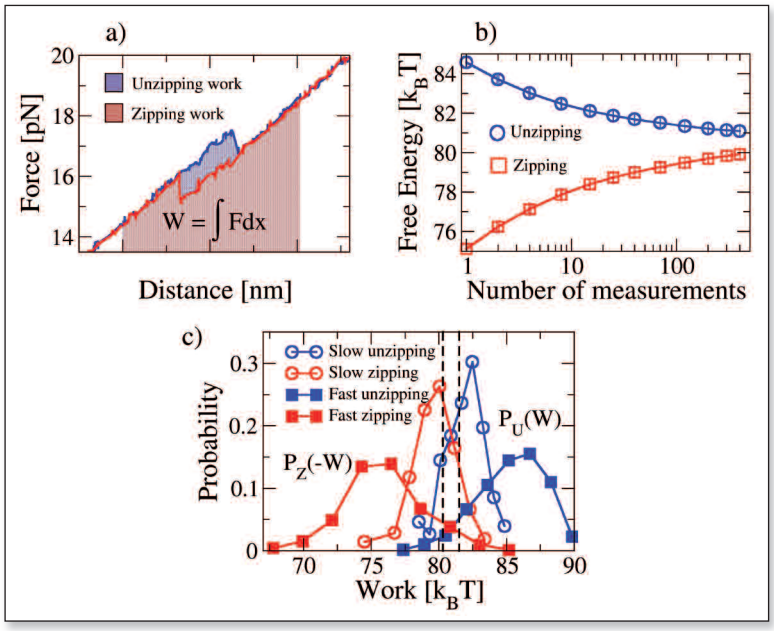


(hairpin, handles and bead) between the initial and final states. After subtracting the contributions (estimated around 20 k_BT) coming from the reversible work performed by the trap to stretch the handles, pull on the bead and stretch the elastic ssDNA released when the hairpin unfolds, we get the free energy of formation of the hairpin at zero force, 61.4±1.0 k_BT and 60.4±1.0 k_BT for the unzipping and zipping trajectories, respectively. Both cases agree well with free energies obtained from melting experiments.

Figure 4b shows the convergence of the JE with the number of measurements for both the unzipping and zipping experiments. In 1999, G. E. Crooks derived a more general relation containing the JE as a particular case and improving the mentioned slow convergence. The key point of the Crooks Fluctuation Relation (CFR) is to consider a given perturbation and its time reversed (unzipping and zipping respectively, in our case). The main result is that the free energy difference corresponds to the work value equally probably in both processes. It can be seen in figure 4c that the crossing point between the two histograms remains the same for different pulling speeds and the numbers agree with the JE.

Various FT have been demonstrated during the past two decades and experimentally tested in different systems like electrical oscillators or hydrodynamic systems [14, 15]. They have been also extended to quantum systems, which are subject to the uncertainty principle and face the intriguing difficulty of measuring work along individual experiments (where the notion of trajectory is not defined) without perturbing them. Future developments consider using FT to recover free energies in more complex situations (*e.g.*, binding free energies of proteins and other molecules or free energies of non-native states).

FT are applicable to small systems where energy fluctuations with respect to the average behaviour (of thermal or quantum origin) are big enough to be measured. The most remarkable examples of such a class of systems are found inside living organisms. Surrounded by water (and many other molecules), cells contain plenty of molecular machines and biomolecules operating out of equilibrium in a highly noisy and crowded environment. FT might provide a theoretical framework to understand how the complexity of life has emerged in such a noisy and irreversible world governed by fundamental laws that are time reversible. ■



▲ FIG. 4: a) The total work is shown as the crossed area below the FDC. b) Convergence of the free energy using the JE as a function of the number of measurements, obtained with the unzipping (blue) and zipping (red) trajectories. c) Work histograms for hairpin in fig. 2b, obtained at two different pulling speeds (60 and 180 nm/s). The crossing point remains approximately constant while dissipation increases with the speed.

About the authors

Anna Alemany graduated in physics in 2007. She is currently doing her PhD at the University of Barcelona under the supervision of Prof. F. Ritort. She is mostly interested in statistical physics with special emphasis on fluctuation theorems applied to single molecule experiments. She is currently doing unzipping experiments on DNA and RNA hairpins using optical tweezers to investigate questions related to the thermodynamics and kinetics of force-induced molecular folding and unfolding.

Felix Ritort is Professor in condensed matter physics at the University of Barcelona (Spain). His PhD received in 1991, was on spin glasses. He has made several contributions to the field of statistical physics of disordered systems, nonequilibrium systems and more recently to biophysics. Currently he runs a single molecule lab in Barcelona where he investigates questions related to the nonequilibrium physics of small systems.

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