Unfolding single RNA molecules: bridging the gap between equilibrium and non-equilibrium statistical thermodynamics

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Abstract. During the last 15 years, scientists have developed methods that permit the direct mechanical manipulation of individual molecules. Using this approach, they have begun to investigate the effect of force and torque in chemical and biochemical reactions. These studies span from the study of the mechanical properties of macromolecules, to the characterization of molecular motors, to the mechanical unfolding of individual proteins and RNA. Here I present a review of some of our most recent results using mechanical force to unfold individual molecules of RNA. These studies make it possible to follow in real time the trajectory of each molecule as it unfolds and characterize the various intermediates of the reaction. Moreover, if the process takes place reversibly it is possible to extract both kinetic and thermodynamic information from these experiments at the same time that we characterize the forces that maintain the three-dimensional structure of the molecule in solution. These studies bring us closer to the biological unfolding processes in the cell as they simulate in vitro, the mechanical unfolding of RNAs carried out in the cell by helicases. If the unfolding process occurs irreversibly, I show here that single-molecule experiments can still provide equilibrium, thermodynamic information from non-equilibrium data by using recently discovered fluctuation theorems. Such theorems represent a bridge between equilibrium and non-equilibrium statistical mechanics. In fact, first derived in 1997, the first experimental demonstration of the validity of fluctuation theorems was obtained by unfolding mechanically a single molecule of RNA. It is perhaps a sign of the times that important physical results are these days used to extract information about biological systems and that biological systems are being used to test and confirm fundamental new laws in physics.

Introduction

Until recently, all chemical and biochemical processes had been investigated by taking advantage of a replica method in which many molecules of one or various species are investigated at the same time. In this macroscopic approach, the changes in the properties of each of the molecules that accompany these transformations, add up to provide a measurable signal. Despite its enormous power, this method – until recently, the one of choice in all research in chemistry and
biochemistry – has its limitations. Specifically, the macroscopic signal obtained in these bulk, macroscopic experiments corresponds to the asynchronous sum of the contributions of each of the molecules in the ensemble and, as such, it represents both a time and an ensemble average of the changes undergone by all molecules. However, very few (if any) of the molecules in the ensemble actually behave as this average. Moreover, interpreting the smooth, continuous changes characteristic of macroscopic observables in terms of equally smooth transformations at the molecular level is both misleading and incorrect. At the molecular level, the chemical processes occur in a stochastic, discrete, and random fashion. Molecules collide and react with each other to form products that can transform back into the initial reactant species. This seemingly random behavior of individual molecules may seem unimportant or (at best) an undesirable property of their microscopic nature. In fact, we may ask: ‘Why do we care about the stochastic, probabilistic behavior of chemical transformations? Isn’t the average sufficient to understand the nature of the chemical reaction?’ The answer is unequivocally no, mainly for two reasons.

The first reason is that the interpretation of macroscopic averages of molecular properties assumes that the distribution of values of these molecular properties is unimodal. If that is not the case, and the molecular properties are distributed in a multimodal fashion, the macroscopic average will lead to gross misinterpretations, as the average values may easily not describe even the mean values of the co-existing populations. This issue becomes even more significant when we attempt to follow a chemical transformation in real time. Chemical and biochemical reactions are often complex processes occurring through multiple steps, alternative pathways, and populating multiple intermediates. Although the nature, lifetime, and obligatory versus alternative participation of these intermediates determine the molecular mechanisms of these transformations, their presence is often difficult to infer from bulk experiments. All molecular populations, even if initially synchronized, will sooner or later de-phase and the signals arising from the individual molecules lose coherence. Therefore, except early on, signals in bulk measurements, are the incoherent sums of the uncorrelated contributions of each molecule in the sample and can provide only the dynamics of the mean of this population (kinetics) but not an accurate picture of the dynamics of the individual molecules.

The second reason has to do with the strains and stresses that are generated in the course of chemical reactions. Since the publication of the classical studies of J. D. van der Waals in 1873, physical scientists have known that many – if not most – properties of matter can be rationalized by the strength and direction of the forces that molecules exert on each other. Even strictly macroscopic phenomena, such as the elasticity and the melting points of solids, the viscosity and boiling points of liquids, or the compressibility of gases, are macroscopic manifestations of the myriad of small interactions between molecules. Similarly, chemists have also known for a long time that chemical affinity results from the attractive interactions between chemical entities. In 1889, Svante Arrhenius proposed that reactions between molecular species follow pathways that involve the formation of some type of strained, largely unstable, high-energy ‘activated’ state whose accessibility along the reaction coordinate controls the rate of the reaction. Despite the enormous importance of these concepts, scientists have not had ways of testing them experimentally.

In recent years (Bustamante et al. 2004), the use of methods to investigate chemical processes at the single-molecule level, has began to change all of this. Single-molecule methods avoid the ensemble average that characterizes bulk methods and make it possible to follow the trajectories of the individual molecules as they undergo their reactions in real time. It is thus possible to characterize the various intermediates in the chemical processes and to follow the alternative
reaction pathways. Similarly, until very recently, chemists and biochemists lacked the methods to investigate the effect of forces or torques on molecules or on their reactions. Methods of direct mechanical manipulation of individual molecules are now making it possible to measure directly the forces holding together molecular structures and to determine the stresses and strains generated in the course of chemical and biochemical reactions. Moreover, it is now possible to exert external forces or torques so as to alter the extent and even the fate of these reactions, and to investigate and reveal the rules that govern the interconversion between chemical and mechanical energy in these processes. First used in studies of the elasticity of individual DNA molecules (Bustamante et al. 1991; Smith et al. 1992), single-molecule manipulation methods constitute a powerful new way to investigate and follow biochemical processes in real time, an area of research that can be rightly called mechanochemistry.

The use of forces and/or torques as experimental variables and the microscopic nature of these experiments pose, in addition, important new theoretical issues such as the need to reformulate the basic equations of physical chemistry in terms of these variables, and the necessity to formulate a theory of the thermodynamics of microscopic systems. In this article, we review the recent application of methods of single-molecule manipulation to mechanically induce the unfolding of single molecules of RNA. We also review some of the recent developments in the use of single-molecule methods to experimentally test newly discovered fluctuation theorems in statistical mechanics. These theorems are of great theoretical importance for they represent direct bridges between the realms of reversible and irreversible statistical mechanics. As we show here, they can be used, in turn, to obtain important equilibrium properties of molecules even when these molecules have been subjected to non-equilibrium processes. For convenience, we introduce the following nomenclature. We will refer to results obtained using single-molecule approaches as experiments having been performed ‘in singulo’ and those obtained using their bulk counterparts as experiments performed ‘in bulk’ or ‘in multiplo’.

The RNA folding problem: reversible unfolding of RNA molecules in singulo by force

RNA molecules must fold into specific 3D shapes to perform their structural and catalytic functions. However, unlike proteins, the stabilities of the secondary interactions in RNA are largely independent of the tertiary context in which they are found in the folded structure (Tinoco & Bustamante, 1999). Energetically, this statement is equivalent to assert that the energy of the molecule is separable as the sum of secondary interactions and tertiary contacts, plus a very small term (mostly negligible) due to the interference between these two terms. Thus, if in addition to the empirical thermodynamic parameters already available for the robust prediction of RNA secondary structure we had those corresponding to tertiary contacts, it would be possible, in principle, to generate algorithms to predict the overall folding of any RNA molecule from its sequence. Such ‘aufbau’ algorithms could use first thermodynamic data of secondary interactions to predict the secondary folding of the molecule, and then utilize the data on tertiary contacts to obtain its most likely fold in 3D space. The ability to predict the structure of RNA molecules from their sequence is of great importance in molecular biology and biomedicine.

Solving the RNA folding problem requires knowledge of the thermodynamic and kinetic parameters involved in forming Watson–Crick base pairs, base triples, loops, pseudo-knots, and other secondary and tertiary structures. We have used optical tweezers to unfold the P5abc domain (Fig. 1b, right) of the T. thermophila ribozyme, whose structure is stabilized by both secondary and
numerous Mg$^{2+}$-dependent tertiary contacts (Liphardt et al. 2001). The experimental arrangement can be seen in Fig. 1a where one RNA molecule is tethered between two plastic beads.

The results of these experiments were compared with those obtained on a mutant (P5ab) (Fig. 1b, left) that cannot form Mg$^{2+}$-dependent tertiary contacts. When kept under constant tension, these mutants displays bi-stability, i.e. the molecules can be followed as they fluctuate back and forth between their folded and unfolded states in real time. When the molecules unfold, the instrument moves the beads apart to prevent the tension in the molecule to drop from its pre-set value. When the molecule refolds, the beads are brought together to prevent the force from increasing. By plotting the distance between the beads in time, it is possible to follow the equilibrium between the two states of the molecule as shown in Figure 2. This figure displays traces of the distance between the beads, i.e. the length of the molecule ($y$ axis) as a function of time ($x$ axis) for six different values of the pre-set forces.

At every pre-set tension, the inverse of the average lifetimes of the molecule in the unfolded state (long distance) and the folded state (short distance) yield the forward and reverse rate coefficients for the folding and unfolding of a single RNA molecule. By changing the pre-set tension, we can obtain these rate coefficients as a function of force, the equilibrium constant for the folding process (as the ratio between the coefficients) at every pre-set tension, and the position of the transition state along the reaction coordinate.

The presence of Mg$^{2+}$ modifies substantially the force-extension dependence of P5abc (but not of P5ab) making it possible to distinguish secondary from tertiary interactions in P5abc. In particular, it is possible to distinguish a kinetic intermediate during unfolding of the P5abc molecule. The distance to the transition state for P5ab is found to be $\sim 11$ nm along its reaction coordinate (the end-to-end distance of the molecule), or roughly halfway between the folded and the unfolded state. This molecule, capable of making only secondary interactions is, thus, elastic and mechanically compliant. In contrast, the distance to the transition state for P5abc...
which can attain both secondary and tertiary structures is only $1.6\text{ nm}$, indicating that the presence of tertiary interactions makes the molecule harder, requiring higher forces to unfold, but also more brittle, indicating that a relatively small deformation is sufficient to commit the reaction to the unfolded state. This result of the effect of tertiary interactions on the distance to the transition state is quite general. Globular proteins, for example, have been found to have very short distances to the transition state along mechanical unfolding coordinates.

Reconstructing the unfolding intermediates of group I intron ribozyme from *T. thermophila*

Many regulatory biological processes in the cell such as mitochondrial import, the progress of a messenger RNA through the ribosome, the unfolding of RNA by helicases, and spliceosome activity, involve controlled mechanical deformation and unfolding of RNA. Until very recently, it has proven difficult to investigate the response of RNA molecules to locally applied mechanical forces. We can use direct mechanical manipulation of individual RNA molecules to address this issue, and answer questions such as: What are the kinetic intermediates in the unfolding of a complex RNA molecule? What are the magnitudes of the mechanical kinetic barriers encountered by the molecular machines (helicases, ribosomes, etc.) that unfold RNA molecules *in vivo*? How do these magnitudes depend on the unfolding rates? Where are the barriers located within the RNA molecules, and what structures or interactions generate the barriers? Seeking answers to all these questions, we attached molecules of the L21 variant of the group I intron ribozyme from *T. thermophila* (Fig. 3a) to polystyrene beads with RNA-DNA hybrid ‘handles’, and used optical tweezers to consecutively unfold and refold the molecules under tension (Onoa *et al.* 2003). Mechanical unfolding trajectories for individual molecules display eight intermediates (Fig. 3b) corresponding to discrete kinetic barriers opposing mechanical unfolding with lifetimes of seconds and rupture forces between 10 and 30 pN.
Barriers are Mg\(^{2+}\)-dependent and we determined their location and the intra- and inter-domain interactions they involve. Several barrier structures are found to be very ‘brittle’, that is, breaking them requires application of high forces but small (1–3 nm) deformations of the structure which, as indicated above, is a signature of the mechanical response of molecules possessing not only stabilizing secondary but also tertiary interactions. Perhaps more importantly, by mutating specific contacts or by passivating them with the use of small oligonucleotides, we have been able to map the location of the kinetic barriers to mechanical unfolding in the complete molecule and to recognize up to eight unfolding intermediates in the process.

Moreover, because barrier crossing is always a thermally activated process, it takes place stochastically, leading to variable unfolding paths each time the molecule is pulled. By pulling the molecule many times, we were able to determine the relative frequency (probability) that the molecule would unfold following one or an alternative unfolding pathway, essentially mapping the potential energy surface over which the molecules diffuse during their mechanical unfolding (Fig. 4). In this figure, the thickness of the arrows is proportional to the probability that the molecule follows one or an alternative pathway.

**Recovering equilibrium information from non-equilibrium measurements: experimental test of Jarzynski’s equality**

The importance of thinking small

Strictly speaking, thermodynamics describes the energy exchange processes of macroscopic systems: liquids, magnets, superconductors, and even black holes, comply with its laws. In macroscopic systems the observed behavior is reproducible and fluctuations (deviations from the typically observed or average behavior) are small. It is only in some special conditions that thermal fluctuations produce readily detectable consequences in macroscopic systems.
Well-known examples are the light scattered from the sky (which is ultimately responsible for its color) or the opalescence of light in a fluid at the critical point.

As the system’s dimensions decrease, fluctuations away from equilibrium begin to dominate the system’s behavior. In particular, in non-equilibrium small systems thermal fluctuations can lead to observable large deviations from their average behavior. Systems of this type abound in
nanotechnology, where motors with dimensions of <100 nm are being built, and inside the cell, where the biological function and efficiency of many molecular machines are determined by their size.

Theoretical background: the fluctuation theorems (FTs)

Non-equilibrium systems are characterized by irreversible heat losses between the system and the environment (generically called here ‘the thermal bath’). Recent developments towards a unified treatment of arbitrarily large fluctuations in small systems are embodied in FTs, which relate the probabilities of a system exchanging a given amount of energy with the thermal bath in a non-equilibrium process (for a review, see Evans et al. 2002 and Bustamante et al. 2005).

Various FTs have been reported, and differ by the details of the system’s dynamics (stochastic versus deterministic), the nature of the thermostat, and the initial conditions (equilibrium or non-equilibrium steady state). A parallel development began in 1997 with the report of a non-equilibrium work relation by Jarzynski (1997), called the Jarzynski equality (hereafter referred to as JE). Consider a system kept in contact with a bath at temperature $T$ whose equilibrium state is determined by the value of the control parameter $x$. Initially, the system is at equilibrium and the control parameter is $x_A$. The non-equilibrium process is obtained by changing $x$ according to a given protocol $x(t)$, from $x(0) = x_A$ to $x(t) = x_B$. The JE states that for any non-equilibrium process that starts at the equilibrium state (A) and ends at the final state (B):

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

where, $\Delta G$ is the free-energy difference between the equilibrium states A and B, and where the average $\langle \ldots \rangle$ is taken over an infinite number of repeated non-equilibrium experiments carried out with the protocol $x(t)$. Frequently, the JE is recast in the form $\langle \exp (-W_{\text{dis}}/k_B T) \rangle = 1$ where $W_{\text{dis}} = W - \Delta G$ is the dissipated work along a given work trajectory.

The exponential average, which is indicated in the JE, implies that $\langle W \rangle \geq \Delta G$ or $\langle W_{\text{dis}} \rangle \geq 0$ which is the statement of the second law of thermodynamics in terms of free energy and work. An important consequence of the JE is that, although on average $W_{\text{dis}} \geq 0$, there must always exist non-equilibrium trajectories with $W_{\text{dis}} \leq 0$ for the equality to hold. These trajectories, sometimes referred to as ‘transient violations of the second law’ (a misnomer, since the second law does not apply for transient processes but for time averages or for ensemble averages), stand for large fluctuations in the work that ensure that the microscopic equations of motion are time-reversal invariant. The JE is remarkable, for it implies that: it should be possible to obtain the equilibrium free energy change, $\Delta G$, experienced by a system in a given process between an initial and a final equilibrium state, from the irreversible work performed to take that system between those same states. Moreover, it states that such a task may be possible even when the process is carried out arbitrarily far from equilibrium (Jarzynski, 1997). As such, this result and the associated FTs discovered since, are of fundamental importance for they represent relationships that bridge the realms of equilibrium and non-equilibrium statistical mechanics.

Experimental test of the JE

We have recently carried out an experimental test of the JE by mechanically stretching a single molecule of RNA reversibly and irreversibly between two conformations (Liphardt et al. 2002).
We recognized (see below) that the validity of the JE depends on the existence of fluctuations for which the $W_{\text{dis}} \leq 0$, and that these fluctuations while rare for large macroscopic systems are reasonably probable for a microscopic system such as a single molecule. Thus, it may be possible to test the validity of the equality when the number of work trajectories (numbers of pulling experiments performed on the molecule) needed to obtain the exponential average is finite and attainable under normal experimental conditions. Indeed, we found that application of this equality to the irreversible work trajectories of the unfolding of single RNA molecules recovers the free-energy profile of the stretching process to within $\pm k_B T/2$ of its best independent estimate, i.e. the average reversible work to unfold the molecule (see Fig. 5).

In Figure 5a, we plot the average work dissipated during the mechanical unfolding a single molecule of P5abc at three different loading rates (blue = 2–5 pN/s, green = 35 pN/s, and red = 52 pN/s) as a function of the reaction coordinate, the end-to-end-distance of the molecule. (b–d) The corresponding histograms of the dissipated work for the three pulling rates and for the different extension of the molecules. The color code is that of panel (a).

![Graph showing average work dissipated during unfolding](image)

**Fig. 5.** (a) Average work dissipated during the unfolding of the P5abc molecule at three different loading rates (blue = 2–5 pN/s, green = 35 pN/s, and red = 52 pN/s) as a function of the reaction coordinate, the end-to-end-distance of the molecule. (b–d) The corresponding histograms of the dissipated work for the three pulling rates and for the different extension of the molecules. The color code is that of panel (a).
Figure 5(b–d) shows histograms of the dissipated work for the three different loading rates (blue, green and red in ascending order, as before) for three different values of molecular extension. For convenience we analyze here the distributions of dissipated work in panel (d). Note that at the lowest loading rate, the distribution of dissipated work is centered at zero, as expected. The mean of this distribution moves to the right for the intermediate and higher loading rates (green and the red curves, respectively) consistent with the data presented in panel (a). However, note that for these two cases, even though the mean of the dissipated work is positive, there are work trajectories for which the dissipated work is negative (left tails of the distributions). What is the meaning of this negative dissipated work? The answer is at the heart of this important FT. The second law of thermodynamics only requires that the mean (i.e. the ensemble average) of the distribution of the dissipated work must be positive, as we found it to be. The second law, however, does not prescribe that individual trajectories cannot behave differently. In fact, every once in a while, during the mechanical unfolding of the RNA molecule, a fluctuation occurs in the bath that helps the operator to unfold the molecule. In these rare instances, the work done to unfold the molecule, even when performed at loading rates in which the molecule is taken out of equilibrium, ends up being smaller than the work required to unfold the molecule reversibly at equilibrium. Their difference, i.e. the work dissipated in this process is thus negative. Remember that the work that appears in the JE is the irreversible work = reversible + dissipated work. When the dissipated work is negative, as in the rare instances described above, the magnitude of this irreversible work is smaller in absolute value. Because of the negative exponential average implied in the JE, these smaller work values have a larger statistical weight than the more common larger values. In this way, a few smaller work values can cancel out the contribution of the majority of the trajectories (associated with the positive side of the distribution of dissipated work values). The Jarzynski average can in this way recuperate the free energy (or reversible work) associated with this process.

Although this equality is valid for systems of all dimensions, it is clear that the average implied in Eq. (1) must include enough of these ‘rare’ instances in which the dissipated work is negative, in order for the right-hand side of the equation to converge to the left-hand side and the equality to hold. Fluctuations are extremely small and rare in large macroscopic systems and thus, the possibility of testing this equality experimentally had to rely on the design of an experiment microscopic enough to undergo detectable fluctuations as is the case with the single- molecule unfolding experiments presented here.

The implementation and test of the JE provides the first example of its use as a bridge between the statistical mechanics of equilibrium and non-equilibrium systems. Conversely, the significance of this work for single-molecule studies is that it extends the thermodynamic analysis of single molecule manipulation data beyond the context of equilibrium experiments, making it possible to extract equilibrium information from non-equilibrium mechanical work.

We have also investigated the rate of convergence of the JE, i.e. how many work trajectories must be averaged for the JE to yield a good estimate of the free energy (Gore et al. 2003). We concluded that for processes in which molecules are taken very far from equilibrium, faster convergence algorithms are desired. Thus, we have been investigating the rate of convergence of other FTs derived at the wake of the discovery of the JE. One of these, the Crooks’ Fluctuation Theorem may provide a faster convergence of the work trajectories towards the free-energy values and, thus, allow us to obtain these values from a reasonable number of trajectories even for molecules that are taken very far from equilibrium.
Epilogue

We have seen above that, unlike experiments in multiplo, experiments done in singulo make it possible to determine not just the average of a molecular property, but its complete distribution over possible values. Experimental observables in singulo correspond to the values of the individual trajectories of molecules and not just the ensemble average of these properties. Moreover, new parameters, such as forces and torques, become variables under experimental control in the novel methods of single-molecule manipulation, making it possible to investigate their effect on the rates, extent and fate of chemical reactions.

One place where the behavior and the description of single molecules matter is the interior of the cell. Indeed, many proteins in the cell are present in very few copies. As a result, many essential cellular processes, such as DNA replication, transcription, chromosomal segregation, etc., display the random and stochastic behavior characteristic of single molecules. The study and analyses of individual molecules is, thus, of great importance if we are to understand how the cell accomplishes a spatial and temporal coordination of these functions when many of these are, in fact, ‘buried’ in noise. Once again, extension of methods of single-molecule detection and manipulation inside the cell will likely play a central role in addressing these important questions.

The microscopic nature of the experiments made possible by these novel methods, are especially well suited to investigate the molecular mechanisms that underlie the function of many of the complex assemblies of the cell. Single-molecule manipulation experiments are also ideal to investigate fundamental physical relationships at the interface between equilibrium and non-equilibrium statistical mechanics, using if needed biological molecules as testing tools. The advent of single molecule methods represents, thus, a fertile ground in which the traditional disciplines of physics, chemistry and biology can converge. That these disciplines, for so many years segregated in their own realms, are now ‘informing’ each other, is one of the most welcomed new developments in science, a true signature of our times.

References


