

intrinsic ability of the enzyme RNase to form domain-swapped dimers. The active site of this enzyme includes two amino acids that are essential for catalysis and that reside on opposing domains. Mutating these amino acids separately generated two pools of inactive mutants. Upon domain swapping, these formed a pool of dimers, one in four of which is active — representing the probability of assembly of complementary mutants. The authors then engineered the hinge region to include the insertion of ten glutamine residues. Alone, this is a weakly amyloidogenic sequence; in the proteins that are mutated in polyglutamine-expansion diseases, such as Huntington's disease, stretches of more than 40 glutamines are required for significant aggregation⁸. Remarkably, a mixed assembly that includes the active-site and hinge-insertion mutations results in the formation of amyloid fibres with an enzymatic activity that is indicative of domain-swapped assembly.

Enzymatic activity is the unquestioned hallmark of a defined protein structure. In the founding days of protein crystallography, serious scepticism existed concerning the relevance of crystal structures to biological

function. Then, as now, RNase played a pivotal role in a seminal study⁹ that established that proteins in crystals can be enzymatically active. There is considerably more mystery to be solved in the formation of amyloid fibres — for example, the structural nature of transient intermediates and the basis of their cytotoxicity. The current study⁵ shows us that a molecular basis for these phenomena, with creative perseverance, is ultimately obtainable. ■

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The force required to move a large, slow-moving plunger is proportional to its speed; all the mechanical work performed is lost, irretrievably, to the environment as heat. At a given speed, the work required to move the plunger between two well-defined states is always the same: viewed microscopically, the resistance to the motion is just the statistically averaged effect of many collisions between the plunger and the fast-moving molecules of the fluid.

If the plunger is very small, however, this average is performed over far fewer collisions, and the random, brownian movements of the plunger become significant. When an excess of molecular collisions occurs in the direction in which the plunger is moving, the work done by the operator is reduced. This work can on rare occasions be reduced to zero, or even become negative — meaning that the system performs work on the operator. The mechanical work needed to move a small plunger is no longer a precise, deterministic variable; rather, it is described by a statistical distribution.

The CFT stipulates that the probability of exerting a given amount of mechanical work on the plunger in the conventional, forward process, divided by the probability of getting the same amount of work back from the reverse process, depends exponentially on the amount of work lost to the environment. This implies that the likelihood of dissipating virtually no work is the same in both the forward and reverse directions. Because the amount of work dissipated increases as a system gets larger, in a sufficiently small system the likelihood that 'dissipation-free' events will occur becomes large enough for them to be observed directly.

Collin *et al.*¹ studied the mechanical unfolding and refolding of individual RNA molecules, applying and measuring forces using optical tweezers — a focused laser beam that acts as an ultra-sensitive spring to trap and hold small particles. The two ends of an RNA molecule, folded into junctions and hairpin shapes, were each linked to a small bead, one held by a micropipette and the other by the optical tweezers. In each experimental cycle,

BIOLOGICAL PHYSICS

Rare returns on lost effort

Wesley P. Wong and Evan Evans

How does the size of a system affect its thermodynamic irreversibility? A deft experiment that observes the unfolding and refolding of a single molecule of RNA provides insights into the question at a small scale.

When vigorously stirred, a cup of coffee will heat up; a cup of hot coffee, however, will never stir a spoon (Fig. 1). This type of irreversibility is a cornerstone of macroscopic thermodynamics. But does it still hold when the spoon is extremely small? Newly discovered relationships between heat, work and energy have revealed unexpected features of small systems when they are driven far from equilibrium — relationships that are becoming increasingly relevant as experiments probe ever-tinier systems, including the nanoscale machinery of living cells. On page 231 of this issue, Collin *et al.*¹ describe a single-molecule experiment that not only validates one prominent recent postulate, known as the Crooks fluctuation theorem, but also provides a new method for quantifying the difference in equilibrium free energy — the useful work expected to be extracted from a system — between two biomolecular states.

The Crooks fluctuation theorem² (CFT) describes the exchange of energy between a system and its environment in forward and reverse processes. It generalizes the so-called Jarzynski equality³, which relates equilibrium

free energy to the work done in multiple, non-equilibrium measurements. The application of the CFT to a single-molecule experiment can be understood by considering an idealized 'dashpot' — a plunger that can move through a viscous fluid held at a constant temperature.



Figure 1 | Stirring stuff. A paradigm of thermodynamic irreversibility: the world's largest cappuccino.

S. RELLANDINI/REUTERS

the beads were moved apart to produce an unzipping or unfolding transition, and then moved back together to allow refolding.

By integrating the force over the change in length of the molecule through many cycles, Collin and colleagues¹ obtained separate statistical distributions for the work done on the molecule during the forward and reverse transitions. They used these to verify that the CFT holds. By finding the energy at which the two distributions crossed for a range of speeds of bead movement, the authors were able to evaluate the free energy of transition between the folded and unfolded state of an individual RNA hairpin. They also demonstrated the robustness of the CFT by using their analysis to quantify the equilibrium free energy accurately even when the beads were moved apart too fast for the system to respond — in other words, when the system was taken far from equilibrium.

Collin and colleagues' work clearly verifies that the CFT can be applied to small biomolecular systems. But what are its wider implications for single-molecule research? The method provides a unique approach to quantifying the free energy in two-state systems — even in those systems far from equilibrium, where the transition between the two states requires very different amounts of work depending on its direction (a phenomenon known as hysteresis). This is important, as it is often the drifting baseline of a measuring instrument that limits how slowly, and thus how close to equilibrium, an experiment can be performed.

The CFT method is unlikely, however, to be suitable for all molecular systems and probe techniques. Its success in Collin and colleagues' experiments is due in part to certain features of the RNA hairpins and junctions used that made force a relatively smooth function of displacement between the well-defined initial (folded) and final (unfolded) states. Specifically, the free-energy landscape between these states resembles a 'staircase' of small energetic steps of short duration spread over an easily observable distance of many nanometres. The nonlinear, elastic behaviour of the polymer linkers and unfolded RNA chain also ensured that forward and reverse responses could be easily aligned in the presence of instrument drift. These attributes, together with the exquisite sensitivity of the optical tweezers — in regard to both the force applied and the displacement caused — allowed accurate integration for calculating the work during both unfolding and refolding. It also allowed for the subtraction of energetic contributions from the optical tweezers and chemical linkers, a necessary step in precisely determining the free-energy difference of the molecular transition. Extending Collin and colleagues' technique to other biomolecular transitions is thus sure to present fresh challenges.

Even so, this exciting experiment has provided insight into the way that recently developed fluctuation theories can be applied

to transitions in biomolecular systems. Not only does physics teach us new ways to understand single-molecule measurements, but such measurements also feed back into theory, extending our knowledge of thermodynamics to systems that are small and out of equilibrium. Stir a cup of coffee with a small enough spoon, and the coffee might just stir you. ■

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EVOLUTIONARY GENETICS

Microarrays and species origins

Roger Butlin and Cally Roper

Whole-genome arrays have been used to reveal small islands of genetic differentiation in *Anopheles* mosquitoes. Analysis of these regions will identify genes involved in the initial stages of speciation.

What are the genetic modifications that underlie the first steps in the origin of new species? The use of microarrays to make whole-genome comparisons between populations that can interbreed, but only to a limited degree, provides a fresh approach to the question. As reported in *PLoS Biology*, Turner *et al.*¹ have applied this methodology to the malaria-carrying mosquito, *Anopheles gambiae*. They have identified three small regions, less than 1.5% of the genome, that contain critical genes for the initiation of speciation.

In the classic model of speciation, two populations diverge during a period of geographical separation and cannot exchange genes when the physical barrier is removed. In this case the two genomes accumulate differences uniformly. There is, however, often gene flow during the period of divergence, or after secondary contact if reproductive isolation is incomplete. In these cases, genetic differentiation becomes unevenly distributed across the genome as gene flow erodes differences between species, leaving islands of differentiated sequence only around the genes responsible for reproductive isolation². This pattern has been nicely illustrated by the sequencing of multiple genetic loci in closely related species pairs of the fruitfly *Drosophila*^{3,4}, and by surveying differentiation at many anonymous markers, for example in the winkle, *Littorina saxatilis*⁵.

Whole-genome microarrays are used primarily for studies of gene expression, but they can also be used to detect differences in DNA sequences. Genomic DNA is hybridized to the array and a weak signal indicates a mismatch between the individual's DNA and the probe sequence. This principle has been successfully applied to the genetic mapping of quantitative traits in a plant, *Arabidopsis*⁶. Now, Turner and colleagues have used it to scan the whole mosquito genome for genetic

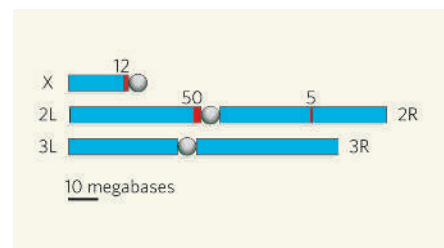


Figure 1 | Differentiated regions in the genomes of the M and S forms of *Anopheles gambiae*.

Anopheles gambiae has three chromosome pairs (X/Y, and 2 and 3, with the two arms of 2 and 3 being designated L and R). Numbers above the differentiated regions, shown in red, are counts of predicted genes within them: among these are genes critical for the initiation of speciation. The small differentiated region on 2R is least strongly supported statistically, perhaps simply because it is small. It may be significant that two of the differentiated regions are close to centromeres (circles).

variants that distinguish two forms of *Anopheles gambiae* known as M and S.

The M and S forms probably began to diverge very recently, perhaps within the past 10,000 years. The M form is restricted to West Africa, where it occurs alongside the S form, and the two interbreed at a low frequency (about 1.2%)⁷. The resulting hybrids are viable and fertile. Fixed differences unique to each species occur in the genes for ribosomal RNAs on the X chromosome⁸. But variable genetic markers elsewhere in the genome show little or no differentiation⁹.

Turner and colleagues' approach involved hybridizing DNA from seven individuals of each form, from Cameroon, to arrays of 142,065 probes each 25 base pairs long. Regions containing concentrations of differences between M and S were detected using a 300-probe sliding window, and just three small regions turned out to be significantly