



Equilibrium Information from Nonequilibrium Measurements in an Experimental Test of Jarzynski's Equality

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10. Improvement per day is for closed-loop minus open-loop hit rate. P values for differences are based on paired t tests. The mean % of each trajectory in the correct octant (as described in Table 1) = $18 \pm 3\%$ (open-loop) and $32 \pm 3\%$ (closed-loop); $P < 0.0001$.
11. The R^2 values are lower than what's usually seen in the literature, because rates were not averaged together across all movements per target. Brain-controlled R^2 values were calculated at the 420-ms window length because this matched the average window length used in the hand-control calculations.
12. "Tuning equation fit," measured by target-averaged R^2 values, was significantly higher during brain-control than hand-control ($\Delta R^2 = R^2_{(\text{brain-control})} - R^2_{(\text{hand-control})} = 0.18 \pm 0.06$), and this difference increased with practice ($d\Delta R^2/\text{day} = 0.004$, $P < 0.0001$).
13. In the earlier arms-free brain-controlled experiments, asymmetries in the tuning functions resulted in widely varying levels of movement accuracy throughout the workspace. Also, becoming more cosine tuned meant that smaller deviations from the mean firing rates were needed to hold the cursor stationary.
14. Monkey M's target hit rate increased by 3% per day ($P < 0.01$), and the mean sequence length increased by 2.5 targets per day ($P < 0.01$). By the last day, the subject was regularly making sequences of 50 to 70 movements without missing.
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18. We thank D. Moran for allowing us to adapt his base virtual reality program; W. Lyn for his recording assistance; and D. Weber for designing the EMG recording system. Supported by a Whitaker Foundation Fellowship, a Philanthropic Education Organization scholarship, and U.S. Public Health Service contract numbers N01-NS-6-2347 and N01-NS-9-2321.

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Equilibrium Information from Nonequilibrium Measurements in an Experimental Test of Jarzynski's Equality

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Recent advances in statistical mechanical theory can be used to solve a fundamental problem in experimental thermodynamics. In 1997, Jarzynski proved an equality relating the irreversible work to the equilibrium free energy difference, ΔG . This remarkable theoretical result states that it is possible to obtain equilibrium thermodynamic parameters from processes carried out arbitrarily far from equilibrium. We test Jarzynski's equality by mechanically stretching a single molecule of RNA reversibly and irreversibly between two conformations. Application of this equality to the irreversible work trajectories recovers the ΔG profile of the stretching process to within $k_B T/2$ (half the thermal energy) of its best independent estimate, the mean work of reversible stretching. The implementation and test of Jarzynski's equality provides the first example of its use as a bridge between the statistical mechanics of equilibrium and nonequilibrium systems. This work also extends the thermodynamic analysis of single molecule manipulation data beyond the context of equilibrium experiments.

Irreversible processes as diverse as mechanically induced protein unfolding, the fracture of stressed materials, and the sudden formation of crystallization nuclei all involve the time evolution of states far removed from equilibrium. To characterize these nonequilibrium states, it is generally necessary to specify numerous details of the system and its surroundings. By contrast, reversible processes are idealizations in which a system passes only through a succession of equilibrium

states, which can be described completely with only a few variables such as pressure and temperature. Reversible processes are powerful tools in thermodynamics because they make it possible to relate the measured heat and work to the thermodynamic state variables. Yet many processes in nature relax to equilibrium only very slowly, precluding quasi-reversible experiments and thus preventing measurement of the thermodynamic state variables. Solving the problem of recovering thermodynamic variables from irreversible experiments remains one of the unfinished tasks in thermodynamics.

It follows from the laws of thermodynamics, first formulated in the early 19th century, that the increase in Gibbs free energy ΔG and the mean work $\langle w \rangle$ needed to bring about that increase are related by $\Delta G \leq \langle w \rangle$. The equal-

ity holds when a process is carried out reversibly, and the inequality holds otherwise. In 1951, Callen and Welton realized that for any system that remains near equilibrium, the energy dissipated is proportional to the system's fluctuations (I). With this fluctuation-dissipation relation, researchers acquired a better estimate of ΔG for irreversible processes: $\Delta G \approx \langle w \rangle - \beta \sigma^2/2$, where σ is the standard deviation of the work distribution and $\beta^{-1} \equiv k_B T$ (where T is absolute temperature and k_B is Boltzmann's constant) (2–4). Unfortunately, this ΔG estimate is valid only in the near-equilibrium regime, and so it was thought that free energies could only be obtained for processes remaining close to equilibrium.

This state of affairs changed in 1997, when Jarzynski derived an equality (5–8) that relates the free energy difference separating states of a system at positions 0 and z along a reaction coordinate, $\Delta G(z)$, to the work done to irreversibly switch the system between two states,

$$\exp[-\beta \Delta G(z)] = \lim_{N \rightarrow \infty} \langle \exp[-\beta w_i(z, r)] \rangle_N \quad (1)$$

where $\langle \rangle_N$ denotes averaging over N work trajectories, $w_i(z, r)$ represents the work of the i th of N trajectories, and r is the switching rate (9). The mechanical work $w_i(z, r)$ required to switch the system between positions 0 and z under the action of a force F is

$$w_i(z, r) \approx \int_0^z F_i(z', r) dz' \quad (2)$$

where $F_i(z', r)$ is the external force applied to the system at position z' with switching rate r (10). Equations 1 and 2 state that the free energy change for a reaction can be determined by averaging Boltzmann-weighted work values obtained from repeated irreversible switching of the system (11, 12). Unlike most expressions relating equilibrium and nonequilibrium statistical mechanics, Jarzynski's equality holds for systems driven arbitrarily far from equilibrium [for other relations that are valid in the far-from-equilibrium regime, see, e.g. (13–20)].

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Despite its possible application to systems where the equilibrium regime is not accessible, Jarzynski's equality has not previously been tested experimentally. Here, we carry out such a test and demonstrate the practical application of the equality by using it to extract ΔG values from nonequilibrium single molecule pulling experiments. Specifically, we compare the performance of three different ΔG estimates: the average work $W_A = \langle w \rangle_N$, the fluctuation-dissipation estimate $W_{FD} = \langle w \rangle_N - \beta\sigma^2/2$, and the estimate obtained from Jarzynski's equality, $W_{JE} = -\beta^{-1} \ln \langle \exp(-\beta w) \rangle_N$ (21).

Several requirements must be met to test Jarzynski's equality. First, the equilibrium and nonequilibrium regimes must both be experimentally accessible. Second, although Jarzynski's equality applies in theory to systems of any size driven arbitrarily far from equilibrium, the experimentally reachable number N of experiments limits the use of Eq. 1 to systems in which the standard deviation of the work values is not much greater than $k_B T$ (5, 10). Thus, the system must be microscopic. Third, measurement error must be kept sufficiently low over a large number of switching trajectories.

Our criteria for verification of the equality are as follows. First, Jarzynski estimates W_{JE} obtained from experiments done at different nonequilibrium switching rates must coincide to within experimental error. This criterion tests the validity of Jarzynski's equality under perturbations of different strengths. Second, the various W_{JE} estimates must also coincide to within experimental error with our best independent estimate of ΔG —the mean work of reversible quasi-static switching, $W_{A,rev}$.

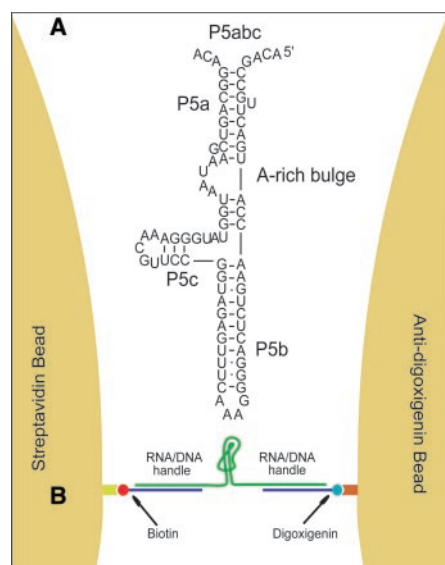


Fig. 1. (A) Sequence and secondary structure of the P5abc RNA. (B) RNA molecules were attached between two beads with RNA-DNA hybrid handles.

Our experimental system is the mechanical unfolding of single RNA molecules derived from the P5abc domain of the *Tetrahymena thermophila* group 1 intron (Fig. 1A) (22). Mechanical unfolding of P5abc is well suited to test Jarzynski's equality because both regimes of interest are experimentally accessible: P5abc unfolds reversibly when stretched slowly and irreversibly when stretched more rapidly. Furthermore, the mechanical unfolding reaction follows an externally imposed and well-defined reaction coordinate, the molecular end-to-end extension z . Individual RNA molecules were attached to 2- to 3- μm polystyrene beads by RNA-DNA hybrid handles and complementary DNA-bead chemistry (Fig. 1B) (23). One bead was held in a force-measuring optical trap (10, 23, 24) and the other bead was linked to a piezoelectric actuator through a micropipette tip. Molecules were stretched by moving the tip bead; the force acting on the RNA was determined by measuring the deflection of the trapping laser beams with position-sensitive photodetectors. Experiments were performed at a temperature of 298 to 301 K.

We unfolded the P5abc domain at slow (2 to 5 pN/s) and fast (34 and 52 pN/s) rates. Each fast unfolding-refolding cycle was immediately followed by one slow cycle. By interleaving fast and slow unfolding-refolding cycles, we could monitor drift of the zero force point in the instrument and of the molecular end-to-end extension, and thus reduce instrumental artifacts in the difference between the work done at the fast and slow rates (25). An RNA molecule was switched N times between the folded and unfolded conformations, and then values for W_A , W_{FD} , and W_{JE} of the fast and slow cycles were computed. Seven independent data sets were collected, each with a different RNA molecule and about 40 unfolding-refolding cycles per switching rate. Data for unfolding-refolding

rates of 2 to 5 pN/s, 34 pN/s, and 52 pN/s are shown in blue, green, and red, respectively, in Figs. 2 and 3.

To confirm that unfolding-refolding of P5abc is reversible at our slowest switching rates, we quantified the mean work difference between the forward and backward curves. In these conditions, P5abc unfolding and refolding curves nearly coincide. Initially, the force-extension curve increases monotonically as the molecular RNA-DNA handles are stretched against entropic elasticity (Fig. 2A, blue). At ~ 10 pN, the RNA molecule begins to unfold. Above ~ 14 pN, the force-extension curve again increases monotonically and is dominated by the molecular handles. Here and below, we consider only 30 nm of the pulling reaction (341 to 371 nm) because handle stretching is reversible at all our switching rates. Accordingly, we place the lower integration limit of Eq. 2, $z = 0$, at 341 nm. At this slow switching rate, the difference between the mean work of forward and backward curves is smaller than $k_B T$ at any position along the pulling coordinate z from 0 to 30 nm ($N = 24$ curves). We estimated the experimental error in our measurements to be $\pm k_B T/2$ and used this value as the threshold beyond which discrepancies between energy estimates are significant. Slow unfolding-refolding of P5abc was experimentally indistinguishable from a reversible process and yielded a ΔG between the folded and unfolded states of $60.2 \pm 1.6 k_B T$.

Next, we performed the same experiment irreversibly, stretching P5abc and the RNA-DNA handles rapidly. As before, the beginning and end of the stretching process are reversible (Fig. 2A, red), demonstrating that the relaxation rate of the handles is rapid relative to all three switching rates (26). By contrast, hysteresis is observed in the middle of the process (Fig. 2A, red; between 9 and

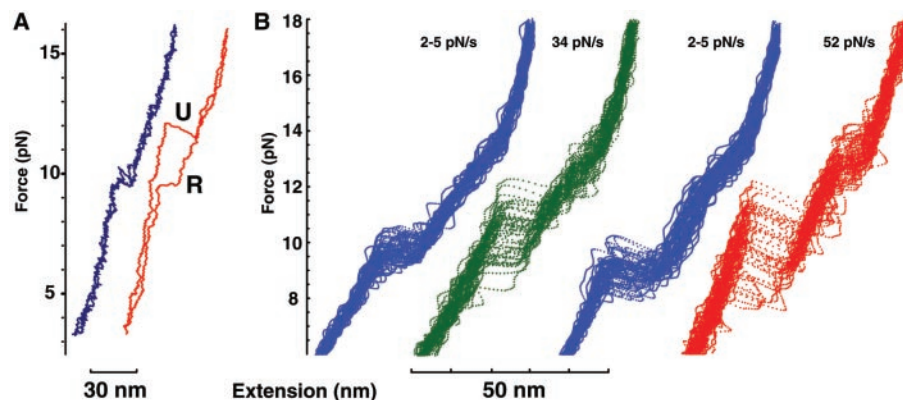


Fig. 2. Force-extension unfolding curves of P5abc at three different switching rates. (A) Typical force-extension unfolding (U) and refolding (R) curves of the P5abc RNA in 10 mM EDTA in reversible (blue, 2 to 5 pN/s) and irreversible (red, 52 pN/s) switching conditions. (B) Two experiments are shown: one in which a molecule was unfolded at rates of 2 to 5 pN/s and 34 pN/s (left pair, blue and green), and another in which the molecule was unfolded at rates of 2 to 5 pN/s and 52 pN/s (right pair, blue and red). Curves (superposition of about 40 curves per experiment) were smoothed by convolution with a Gaussian kernel.

12 pN), illustrating that P5abc RNA unfolding is slow compared to the switching times at 34 and 52 pN/s. As shown in Fig. 2B, at these rapid switching rates (green and red curves) the force-extension curves in the region dominated by P5abc RNA unfolding are higher in force and more broadly distributed than during reversible unfolding (blue curves). However, the refolding curves are slightly lower in force than the reversible curves [on average by 0.3 pN (27)].

The performance of the three different types of ΔG estimates for reversible switching is shown in Fig. 3A. The dissipated work w_{diss} is defined as the difference between the actual work performed on the system and the reversible part of that work; solid lines show the mean dissipated work, $\langle W_{\text{diss}} \rangle = \langle W_{\text{A}} - W_{\text{A,rev}} \rangle$, where the brackets now represent averaging over m data sets ($m = 7$) and W still represents averaging over the N pulls within a data set. Dotted lines show $\langle W_{\text{FD}} - W_{\text{A,rev}} \rangle$, the mean difference between the fluctuation-dissipation ΔG estimate, W_{FD} , and the reversible work. Dashed lines show $\langle W_{\text{JE}} - W_{\text{A,rev}} \rangle$, the mean difference between the ΔG estimate from Jarzynski's equality, W_{JE} , and the reversible work. In this reversible case, the ΔG estimate obtained via Jarzynski's equality is no different from the one obtained using the fluctuation-dissipation relation (note the coincidence to within $0.1k_{\text{B}}T$ of the dashed and dotted blue lines). The coincidence of W_{FD} and W_{JE} for the reversible case is consistent with theoretical predictions for near-equilibrium conditions (21).

At these slow switching rates, W_{FD} and W_{JE} both decay gradually with increasing z , leading to underestimation of ΔG by $1.4 k_{\text{B}}T$ at $z = 30$ nm (Fig. 3A). Such ΔG underestimation is large under conditions of slow switching and results from two related effects. First, the longer the switching time, the more low-frequency instrument noise will accumulate during the experiment, increasing the measured standard deviation of work values, σ . Second, the distribution of molecular work values narrows as the RNA molecule is unfolded more slowly, and this increases the relative contribution of low-frequency instrument noise to the measured σ . Because the dominant contribution to W_{JE} comes from values in the lower tail of the work distribution, this and related estimates of ΔG are particularly sensitive to artifacts that increase σ (28).

Figure 3B shows the performance of the three different types of ΔG estimates for irreversible switching. By $z = 30$ nm, irreversible unfolding (solid green and red curves) leads to dissipation of ~ 2 to $3 k_{\text{B}}T$ compared to reversible unfolding (solid blue curve; yellow band, our experimental error of $\pm k_{\text{B}}T/2$). The dissipated work is the energy penalty for switching a system

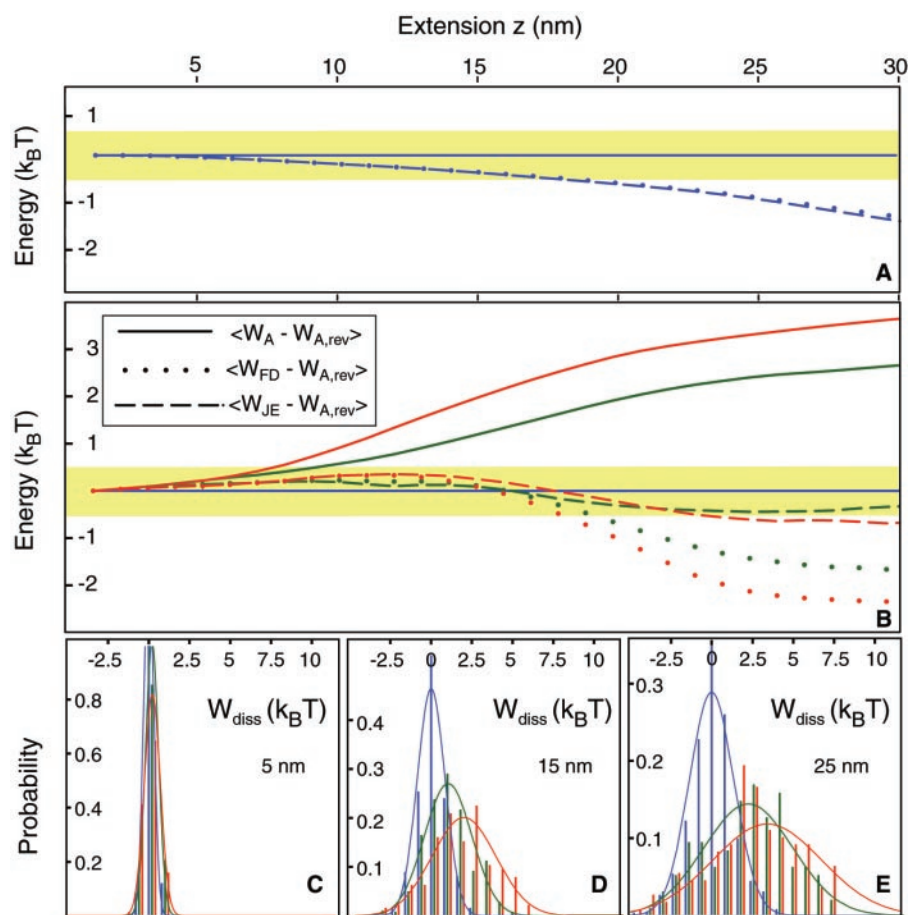


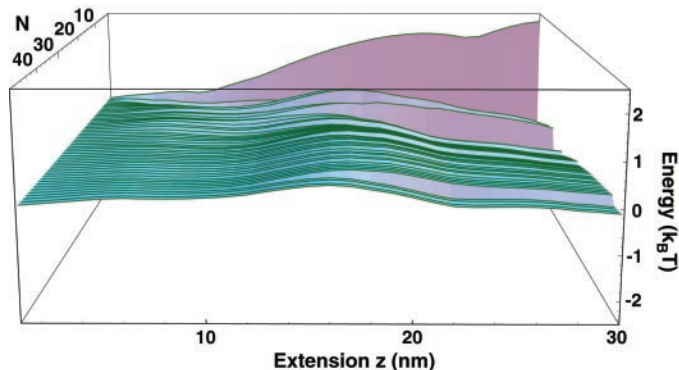
Fig. 3. (A) Estimation of free energy profile from reversible switching ($r = 2$ to 5 pN/s, blue). For each of the seven data sets, we determined the mean work W_{A} , the fluctuation-dissipation ΔG estimate W_{FD} , and the ΔG estimate from Jarzynski's equality, W_{JE} . Next, we subtracted $W_{\text{A,rev}}$ from those energies. Finally, we averaged those differences over data sets, yielding the average dissipated work $\langle W_{\text{A}} - W_{\text{A,rev}} \rangle$ (solid yellow band, $\pm k_{\text{B}}T/2$), $\langle W_{\text{FD}} - W_{\text{A,rev}} \rangle$ (dotted line), and $\langle W_{\text{JE}} - W_{\text{A,rev}} \rangle$ (dashed line). The two ΔG estimates W_{FD} and W_{JE} coincide everywhere to within $0.1k_{\text{B}}T$. Both estimates decrease monotonically with extension, and, by $z = 30$ nm, underestimate the average work by $\sim 1.4k_{\text{B}}T$. (B) Estimation of free energy profile from irreversible switching ($r = 34$ pN/s, green, and 52 pN/s, red). Mean energy differences were computed as in (A). Use of the fluctuation-dissipation relation (dotted lines) yields ΔG to within $k_{\text{B}}T/2$ between $z = 0$ and 18 nm. Beyond $z = 18$ nm, however, W_{FD} underestimates ΔG substantially. By contrast, application of Jarzynski's equality (dashed lines) recovers ΔG to within experimental error ($\pm k_{\text{B}}T/2$) from $z = 0$ to 30 nm. (C to E) Histograms of dissipated work values at $z = 5, 15,$ and 25 nm. Dissipated work values for a given switching rate were pooled. Blue, 272; green, 119; red, 153 dissipated work values. Solid lines: Gaussian with mean and standard deviation of data.

faster than its slowest relaxation rate, and this penalty is largely paid in the central extension range ($10 < z < 18$ nm). The kinetic barrier for unfolding the P5abc molecule is thus located near or within the P5a helix and the A-rich bulge (Fig. 1A). The ΔG estimate from the fluctuation-dissipation relation, W_{FD} , performs well until $z = 18$ nm but then fails (Fig. 3B, dotted curves). However, W_{JE} performs consistently well over the entire extension range. Remarkably, application of Jarzynski's equality (Eq. 1) to work trajectories obtained at the two nonequilibrium switching rates yields ΔG estimates that coincide to within $0.3k_{\text{B}}T$ over the entire extension range (green versus red dashed curves).

Moreover, the difference between the non-equilibrium W_{JE} 's and the reversible work $W_{\text{A,rev}}$ (solid blue curve) is less than $0.6k_{\text{B}}T$ regardless of switching rate. Both criteria laid out earlier for successful verification of Jarzynski's equality are thus satisfied, except near the end of the reaction ($z = 30$ nm), where W_{JE} , 52 pN/s underestimates ΔG by $0.6 k_{\text{B}}T$.

As shown in Fig. 3B, W_{FD} and W_{JE} yield different estimates of ΔG in irreversible conditions (compare dotted and dashed curves). The discrepancy between W_{FD} and W_{JE} at 34 and 52 pN/s suggests that P5abc unfolding occurs far from equilibrium at these rates. The fluctuation-dissipation relation is applicable only in the near-equilibrium limit, where there is a simple

Fig. 4. Convergence of the Jarzynski estimate W_{JE} toward ΔG as a function of the number of pulling cycles N . Switching rate, 34 pN/s. Shading reveals the energy difference between successive $W_{JE} - W_{A,rev}$ profiles. A single molecule was unfolded 47 times, and W_{JE} was updated with each new work trajectory. $W_{A,rev}$ was kept constant at the average work over all 47 reversible curves. By $N \sim 40$, W_{JE} reveals the ΔG profile to within experimental error. At the end of the experiment ($N = 47$), W_{JE} is still converging toward ΔG ; additional work trajectories would further improve recovery of ΔG .



linear relation between dissipation and thermal fluctuation. Jarzynski's equality, however, makes no such assumptions and holds even in the far-from-equilibrium regime. Violent perturbation of the system, and departure from the near-equilibrium regime, should therefore lead to discrepancies between W_{FD} and W_{JE} , as are indeed observed (Fig. 3B). In the middle and end of the switching reaction, the estimation error of ΔG by W_{JE} is one-fourth that of W_{FD} , and thus W_{JE} recovers ΔG to within experimental error of $\sim \pm k_B T/2$ over the entire extension range. The equilibrium ΔG for P5abc unfolding obtained from application of Eq. 1 to these nonequilibrium experiments is $59.6 \pm 0.2 k_B T$ (29), in excellent agreement with theoretical predictions and our earlier equilibrium single-molecule measurements (23).

One feature of W_{JE} helps explain the strengths and limitations of Jarzynski's equality. For the nonequilibrium experiments, W_{JE} overestimates $W_{A,eq}$ by $< 0.4 k_B T$ at those extensions where the degree of work dissipation is largest ($z = 14$ nm, Fig. 3B, green and red dashed lines). Figure 4 shows the convergence of W_{JE} to $W_{A,rev}$ for one data set as the number of curves N included in the calculation of W_{JE} is increased from 1 to 47. The overestimate here originates from the use of Jarzynski's equality with a finite (and low) number of pulls N . Generally, the more work dissipated, the larger N needs to be to converge to ΔG (10). Thus, in the region where most work is dissipated, the convergence of W_{JE} toward ΔG is slow and the effect of finite N is seen most clearly (Fig. 4, $z = 14$ nm).

How is it that a particular form of averaging (Eq. 1) recovers the equilibrium ΔG of a reaction from nonequilibrium work distributions? The answer can be found in histograms of the work dissipated during the pulling of the molecule at the three switching rates (Fig. 3, C to E). The means of the distributions for slow switching are centered at zero, by design (Fig. 3, C to E, blue). The mean and standard deviation of

the dissipated work distributions increase with switching rate r and position z along the pulling coordinate (Fig. 3, C to E, green and red). The mean work increases with r because more work is dissipated as a result of friction when the molecule is unfolded more rapidly. The standard deviation of the dissipated work values increases with r for the following reason. Before external perturbation, the molecule is at equilibrium with the thermal bath and samples a Boltzmann distribution of energy states; at higher switching rates, the molecule relaxes less during the reaction, and therefore the spread of the irreversible work distribution more closely reflects the spread of initial energies (30). The standard deviation thus increases with r and all distributions display a tail of negative dissipated work values regardless of switching rate (Fig. 3, C to E). These smaller work trajectories are weighted more by exponential averaging (Eq. 1). Jarzynski's equality asserts that a balance is maintained between the irreversible work trajectories with positive dissipated work values and those with negative ones such that $\langle \exp(-\beta w_{diss}) \rangle = 1$. Therefore, the increases in mean and width of the work distributions cancel out, regardless of how quickly a reaction is performed, yielding ΔG independently of the switching rate. Application of Jarzynski's equality ultimately reduces to the problem of sampling the rare trajectories in the lower tails of the work distributions.

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$$\Delta G = \sum_{n=1}^{\infty} (-\beta)^{n-1} \omega_n / n!$$

where ω_n is the n th cumulant of work values (3). Keeping only the first term of the expansion yields $\Delta G = W_A$ and is valid at equilibrium. Keeping the first two terms of the expansion yields $\Delta G = W_A - \beta \sigma^2 / 2$, which corresponds to W_{FD} and is valid when all higher ($n > 2$) cumulants vanish. This is the case when the work distribution is Gaussian, as expected in the near-equilibrium regime from the central limit theorem (3). For large enough N ,

$$W_{JE} = \sum_{n=1}^{\infty} (-\beta)^{n-1} \omega_n / n!$$

and this is valid arbitrarily far from equilibrium. In the near-equilibrium regime, $W_{FD} = W_{JE}$ and the dotted and dashed lines in Fig. 3A are consequently equal.

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27. J. Liphardt, S. Dumont, S. B. Smith, I. Tinoco, C. Bustamante, data not shown.
28. Because the values in the left tail of the work distribution contribute most to W_{JE} , widening of the standard deviation of work values leads to underestimation of ΔG .
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31. We thank C. Jarzynski, G. E. Crooks, and D. Chandler for valuable discussions and suggestions, and an anonymous referee for suggesting the analytic sampling efficiency estimation. Supported by the Program in Mathematics and Molecular Biology through a Burroughs Wellcome Fund Fellowship (J.T.L.); the Natural Sciences and Engineering Research Council of Canada and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Québec (S.D.); NIH grants GM-10840 and GM-32543; U.S. Department of Energy grants DE-FG03-86ER60406 and DE-AC03-76SF00098; and NSF grants MBC-9118482 and DBI-9732140.

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