Equalities for the Nonequilibrium Work Transferred from an External Potential to a Molecular System. Analysis of Single-Molecule Extension Experiments

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Nonequilibrium work equalities are investigated for a system that experiences both an intrinsic potential energy function, $V(\mathbf{x})$, and an externally controlled time-dependent potential energy function, $u(\mathbf{x},t)$. The work, $w_T(t) = \int d\mathbf{x}_{t'}\cdot\langle(-)\partial u(\mathbf{x}_{t'},t')/\partial \mathbf{x}_{t'}$, is transferred from the external potential to the bare system, defined by $V(\mathbf{x})$, as the system moves along its trajectory, $\mathbf{x}_t$, in response to the forces generated by $V(\mathbf{x}) + u(\mathbf{x},t)$. As observed by Hummer and Szabo, this transferred work, $w_T(t)$, differs in several ways from the so-called accumulated work, $w_A(t) = \int dt'\ \partial u(\mathbf{x}_{t'},t')/\partial t'$, considered by Jarzynski, which represents the work transferred from the external agent controlling $u(\mathbf{x},t)$ into the composite system, defined by $V(\mathbf{x}) + u(\mathbf{x},t)$. The system is assumed to evolve according to an isothermal stochastic dynamics that maps the canonical distribution for the current potential function, $V(\mathbf{x}) + u(\mathbf{x},t)$, into itself in any arbitrarily short time interval. New nonequilibrium work equalities involving $\exp(-\beta w_T(t))$ are derived and compared with related equalities involving $\exp(-\beta w_A(t))$. Whenever the external potential initially vanishes, an average over all canonically weighted initial conditions and subsequent trajectories gives $\langle \exp(-\beta w_T(t))\rangle = 1.0$, even though $\langle w_T(t)\rangle$ is typically positive and time dependent. For a reversible process, $\langle w_T^{rev}\rangle$ is the work required to achieve a particular fluctuated macrostate of the bare system and governs its relative probability. In the case of single-molecule extension experiments, the external potential function, $u(z(\mathbf{x}),t)$, typically depends on the system coordinates via an extensional parameter $z(\mathbf{x})$. The relevant nonequilibrium work equality involving $w_T(t) = \int_{z_a}^{z_b} dz_{t'}(-)\partial u(z_{t'},t')/\partial z_{t'}$, for cases wherein both initial and final extensions are specified, is derived under conditions pertaining to the experiments of Liphardt et al. This new equality is shown to agree rather well with their experimental results. This new equality is also contrasted with the somewhat different relation conjectured by Liphardt et al., the conditions required for validity of the latter are discussed, and errors arising from its use are estimated to be small. A protocol is suggested to determine the free energy profile of the constrained bare system as a function of its extension, and a possible method to assess the adequacy of trajectory sampling simultaneously from the same nonequilibrium force vs extension data is also presented. Finally a connection between the nonequilibrium equality (involving $\exp(-\beta w_T(t))$) for extending the system from z_a to z_b and macroscopic thermodynamics is briefly discussed.

Introduction

Calculation of the equilibrium free-energy difference between two states, wherein the same molecules experience different potential energy functions, is an important problem in statistical mechanics and especially in computational biophysics, where use of free-energy perturbation theory and reversible work protocols is common.[1−7] Direct alteration of the potential energy functions of individual molecules has been accomplished experimentally by single-molecule micromanipulation techniques.[8−35] In numerous instances, it has been possible to vary and measure the stretching force and simultaneous extension of a single molecule. Determining the equilibrium free-energy differences between the stretched-unfolded states and the unstretched-folded states of various molecules is now a topical problem in both theoretical and experimental biophysics.[24,26,29−34,36,37] The common occurrence of hysteresis and nonequilibrium snap-back effects has hindered some efforts to measure *equilibrium* force vs extension curves, from which a reversible work calculation of the free-energy difference can be made. Consequently, there has been much interest in applying a recent equality discovered by Jarzynski, which relates equilibrium free energy differences to the so-called "accumulated work" ($w_A(t)$) along *nonequilibrium* trajectories.[38,39] A related equality derived by Crooks[40] and by Hummer and Szabo[36] relates the equilibrium free-energy *profile* of the molecule as a function of its extension to the so-called "accumulated work" along *nonequilibrium* pulling trajectories.

Jarzynski's equality can be stated in the following way. A system (or molecule) embedded in an isothermal heat bath experiences a total potential energy function

$$V(\mathbf{x},t) = V(\mathbf{x}) + u(\mathbf{x},t) \tag{1}$$

where $V(\mathbf{x})$ is the *intrinsic* potential energy function of the system coordinates, which are specified by the generalized vector $\mathbf{x}$, and $u(\mathbf{x},t)$ is an externally applied potential energy function, which depends explicitly (in a prescribed way) on the time t. In certain circumstances treated below, the external potential takes the form $u(z(\mathbf{x}),t)$, wherein $z(\mathbf{x})$ is an extensional parameter, such as the end−end distance of the molecule in the z direction, which generally depends on the molecular coordinates $\mathbf{x}$. However, the more general form of $u(\mathbf{x},t)$ in eq

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1 will be employed in the developments that are discussed first. The system is assumed to evolve in a stochastic manner (for example by Brownian dynamics[41−43] or Metropolis Monte Carlo[44] evolution) that maps the canonical distribution *for the current potential energy function* into itself without any regard for the prior history of the system or heat bath. Under these conditions, Jarzynski's equality states that

$$\langle \mathrm{e}^{-\beta w_{\mathrm{A}}(t)} \rangle = Z_t^{\mathrm{C}}/Z_0^{\mathrm{C}} = \mathrm{e}^{-\beta(A_t^{\mathrm{C}} - A_0^{\mathrm{C}})} \tag{2}$$

where A_0^{C} and A_t^{C} denote the *equilibrium* Helmholtz free energies for the prevailing *composite* potential energy functions, namely, $V(\mathbf{x}) + u(\mathbf{x},0)$ and $V(\mathbf{x}) + u(\mathbf{x},t)$, respectively, and $A_t^{\mathrm{C}} - A_0^{\mathrm{C}} = -kT \ln[Z_t^{\mathrm{C}}/Z_0^{\mathrm{C}}]$, wherein $Z_t^{\mathrm{C}} = \int \mathrm{d}\mathbf{x} \exp(-\beta(V(\mathbf{x}) + u(\mathbf{x},t)))$ and $Z_0^{\mathrm{C}} = Z_t^{\mathrm{C}}$ for $t = 0$. The accumulated work along the trajectory is defined by

$$w_{\mathrm{A}}(t) = \int_0^t \frac{\partial u(\mathbf{x}_{t'},t')}{\partial t'} \mathrm{d}t' = \int_0^t (u(\mathbf{x}_{t'},t' + \mathrm{d}t') - u(\mathbf{x}_{t'},t')) \tag{3}$$

where $\mathbf{x}_t'$ denotes the system coordinates at time t'. The unusual final expression in eq 3, which lacks a $\mathrm{d}t'$, is a summand form that is most convenient for conversion to a discrete sum, when the time is discretized, as will be done below. The angular brackets in eq 2 denote an average over *all possible* trajectories beginning from *all possible* starting positions $\mathbf{x}_0$, which are weighted according to the canonical distribution, $f_0(\mathbf{x}_0) = \exp(-\beta(V(\mathbf{x}_0) + u(\mathbf{x}_0,0))/Z_0^{\mathrm{C}}$, and ending at all possible final positions. The right-hand side of eq 2 depends only upon the initial and final potential energy functions and is independent of either the rate or route by which the potential energy function is switched from its initial to its final form. Hence, $\langle \exp(-\beta w_{\mathrm{A}}(t)) \rangle$ must also be independent of the rate or route by which the potential energy function is switched. This fact is both remarkable and surprising, because the average value, $\langle w_{\mathrm{A}}(t) \rangle$, generally depends on both the rate and route by which the potential is switched, except in the limit of extremely slow switching, where it becomes independent of either the (slow) rate or route.

It is important to note that $w_{\mathrm{A}}(t)$ is *not* the work transferred from the external potential to the system along the trajectory, which instead is given by[36]

$$w_{\mathrm{T}}(t) = \int F(\mathbf{x}_{t'},t') \cdot \mathrm{d}\mathbf{x}_{t'} = \int (-) \frac{\partial u(\mathbf{x}_{t'},t')}{\partial \mathbf{x}_{t'}} \cdot \mathrm{d}\mathbf{x}_{t'} = (-) \int_{\mathbf{x}_0}^{\mathbf{x}_t} (u(\mathbf{x}_{t'} + \mathrm{d}\mathbf{x}_{t'},t') - u(\mathbf{x}_{t'},t')) \tag{4}$$

where $\mathrm{d}\mathbf{x}_t'$ denotes the change(s) in the generalized vector in time $\mathrm{d}t'$ at t'. Expanding $u(\mathbf{x}_{t'} + \mathrm{d}\mathbf{x}_{t'},t')$ in a Taylor series about $\mathbf{x}_t'$ leads from the last expression in eq 4 to the previous one containing the generalized gradient. The unusual final expression in eq 4 is the most convenient for conversion to a discrete sum.

It is clear from eq 3 that $w_{\mathrm{A}}(t)$ arises from potential function switching at fixed system coordinates, whereas according to eq 4, $w_{\mathrm{T}}(t)$ arises from displacements of system coordinates at a fixed potential function (which is specified by t).

When the potential function is switched from $u(\mathbf{x},0)$ to its final form $u(\mathbf{x},t_{\mathrm{f}})$ in a sufficiently short time t_{f}, the system coordinates remain practically unchanged. In that case, $w_{\mathrm{T}}(t)$ necessarily vanishes, while $w_{\mathrm{A}}(t)$ generally does not. This further demonstrates that $w_{\mathrm{A}}(t)$ and $w_{\mathrm{T}}(t)$ are very different quantities.

In fact, $w_{\mathrm{A}}(t)$ and $w_{\mathrm{T}}(t)$ are both transferred works, but for different systems. $w_{\mathrm{T}}(t)$ is the transferred work for the *bare* or intrinsic system, defined by the intrinsic potential function $V(\mathbf{x})$ but excluding the external potential $u(\mathbf{x},t)$, whereas $w_{\mathrm{A}}(t)$ is the transferred work for the *composite* system defined by $V(\mathbf{x}) + u(\mathbf{x},t)$. The term composite is applied because that system contains an additional potential energy function, $u(\mathbf{x},t)$, that can be independently varied by an outside agent in its surroundings. Because $u(\mathbf{x},t)$ is a purely mechanical (no entropy) quantity, work is transferred from the surroundings (i.e., source of $u(\mathbf{x},t)$) into the composite system simply by altering $u(\mathbf{x},t)$ to $u(\mathbf{x},t + \mathrm{d}t)$ *at instantaneously fixed* $\boldsymbol{x}$. That work is $w_{\mathrm{A}}(t)$. However, for the bare system, $u(\mathbf{x},t)$ is regarded as part of its surroundings, which exerts external forces, $(-)\partial u(\mathbf{x},t)/\partial \mathbf{x}$, on the system coordinates $\mathbf{x}$. Work transfer from the external potential into the bare system generally involves a change in entropy and is not purely mechanical. It requires displacement of the system coordinates at instantaneously fixed external potential function. That quantity is $w_{\mathrm{T}}(t)$.

In certain experiments, the force due to the external potential is more directly available to experimentalists than is the external potential itself. In certain optical tweezer measurements of force vs molecular extension ($z(\mathbf{x})$), the force is measured by deflection of the trapping light beam, when a (tethered) microsphere in the trap undergoes a displacement perpendicular to the trapping beam.[9,29] In other experiments, where magnetic beads are employed, the prevailing force on the bead at the free end of the molecule is reckoned from the distribution of positions of that bead in a transverse direction.[11] Given only the prevailing *forces* exerted by the external potential on the bare system along a trajectory, $w_{\mathrm{T}}(t)$ can be directly calculated, but $w_{\mathrm{A}}(t)$ often requires additional calibration to assess the values of $u(z(\mathbf{x}),t)$ for different system extensions $z(\mathbf{x})$.

The relation between $w_{\mathrm{T}}(t)$ and $w_{\mathrm{A}}(t)$ is ascertained from[36] $\mathrm{d}u = (\partial u(\mathbf{x},t)/\partial t)\ \mathrm{d}t + (\partial u(\mathbf{x},t)/\partial \mathbf{x}) \cdot \mathrm{d}\mathbf{x}$. Rearrangement and integration of this relation from $(\mathbf{x}_0,0)$ to $(\mathbf{x}_t,t)$ yields

$$w_{\mathrm{A}}(t) = w_{\mathrm{T}}(t) + u(\mathbf{x}_t,t) - u(\mathbf{x}_0,0) \tag{5}$$

In cases where the external potential function is altered by changing certain intensive variables, such as an external electric, magnetic, or gravitational field, or by varying an arbitrary external potential function, $u(\mathbf{x}_t,t)$, that intensive variable can be switched arbitrarily rapidly at constant system coordinates. In such cases, $u(\mathbf{x}_t,t) - u(\mathbf{x}_0,0)$ is generally not negligible and $w_{\mathrm{A}}(t)$ differs significantly from $w_{\mathrm{T}}(t)$. In general, $u(\mathbf{x}_t,t) - u(\mathbf{x}_0,0)$ is independent of the system trajectory between $(\mathbf{x}_0,0)$ and $(\mathbf{x}_t,t)$ whereas $w_{\mathrm{A}}(t)$ and $w_{\mathrm{T}}(t)$ both depend on the particular path.

Two potential problems may arise in the application of nonequilibrium work equalities. (1) In certain experiments, only $w_{\mathrm{T}}(t)$, not $w_{\mathrm{A}}(t)$, may be directly available, as noted above and discussed further below. However, until now, no nonequilibrium equality for $\langle \exp(-\beta w_{\mathrm{T}}(t)) \rangle$ has been reported. (2) The experimental or computational data may enable less complete averaging of either $w_{\mathrm{A}}(t)$ or $w_{\mathrm{T}}(t)$ over initial and final positions of all coordinates than is required by Jarzynski's equality. Such incomplete averaging in turn may allow new information to be extracted.

As shown by Hummer and Szabo,[36] for the case when $u(z(\mathbf{x}_t),t)$ depends only upon an extensional parameter ($z(\mathbf{x}_t)$) and the time t, one finds

$$\langle \delta(z(\mathbf{x}_t) - z_{\mathrm{f}}) \mathrm{e}^{-\beta(w_{\mathrm{T}}(t) - u(z(\mathbf{x}_0),0))} \rangle = (Z_0^{\mathrm{in}}(z_{\mathrm{f}})/Z_0^{\mathrm{C}}) = \exp(-\beta A_0^{\mathrm{in}}(z_{\mathrm{f}})) \tag{6}$$

wherein

$$Z_0^{\mathrm{in}}(z_\mathrm{f}) = \int \mathrm{d}\mathbf{x}\, \delta(z(\mathbf{x}) - z_\mathrm{f}) \exp(-\beta V(\mathbf{x})) \tag{7}$$

$$Z_0^{\mathrm{in}} = \int \mathrm{d}\mathbf{x} \exp(-\beta V(\mathbf{x})) \tag{8}$$

The (relative) free-energy profile of the bare system is defined by Hummer and Szabo as[36]

$$A_0^{\mathrm{in}}(z) = -\beta^{-1} \ln(Z_0^{\mathrm{in}}(z)/Z_0^{\mathrm{C}}) \tag{9}$$

where Z_0^{C} is defined following eq 2. The left-hand side of eq 6 is the average *density* of $\exp(-\beta w_\mathrm{T})$ at a particular point z_f in the one-dimensional space of the extensional parameter $z(\mathbf{x}_t)$. Then multiplying both sides of eq 6 by $\exp(-\beta u(z_\mathrm{f},t))$, using $w_\mathrm{A}(t) = w_\mathrm{T}(t) + u(z(\mathbf{x}_t),t) - u(z(\mathbf{x}_0),0)$, and integrating $\mathrm{d}z_\mathrm{f}$ yields eq 2. The $\mathrm{d}z_\mathrm{f}$ integration effectively completes the averaging over all final extensions, removes detailed information about the free energy profile of the bare system, and finally yields the free-energy change of the composite system as the potential function is varied from its initial to its final form.

In the first experimental test of Jarzynski's equality, the particular relation actually tested was a conjectured modification of eq 2 with $w_\mathrm{T}(t)$ in place of $w_\mathrm{A}(t)$ and with fundamentally different averaging than that in either eq 2 or 6. Until now, a valid nonequilibrium work equality pertaining to the experiments of Liphardt et al.[30] has not been reported.

The Questions. The preceding remarks raise several questions.

(1) Can a nonequilibrium equality for $\langle \exp(-\beta w_\mathrm{T}) \rangle$ be derived? Such a relation might help to diminish future confusion in this area.

(2) What is the physical meaning of the *reversible* transferred work, $\langle w_\mathrm{T}^{\mathrm{rev}} \rangle$? In particular, how does $\langle w_\mathrm{T}^{\mathrm{rev}} \rangle$ differ from $A_t^{\mathrm{C}} - A_0^{\mathrm{C}}$ for the composite system or from the free-energy profile of the bare system, $A_0^{\mathrm{in}}(z)$?

(3) What is the appropriate nonequilibrium work equality to analyze the experiments performed by Liphardt et al.?[30] How does this new equality compare with their experimental results, and how does it contrast with the relation conjectured to interpret their data? In these pioneering experiments, the molecule is manipulated via covalently attached microspheres at either end. The extensional parameter $z(\mathbf{x})$ is induced to vary by confining the microsphere at the head end of the molecule in an optical trap, and attaching the microsphere at the tail end of the molecule to a pipet, as shown in Figure 1. The distance ($\zeta(t)$) between the pipet and the trap center is then varied in a completely prescribed manner. In principle, the experiment can be performed by holding the pipet and solution fixed and moving the trap, which then provides the instantaneous pulling force. The molecular extension $z(\mathbf{x}_t)$ is the distance from the pipet to the center of the microsphere at the head end of the molecule. In general, this instantaneous molecular extension, $z(\mathbf{x}_t)$, is not exactly equal to $\zeta(t)$. The external potential energy function (due to the trap) varies harmonically with the displacement of the microsphere from the trap center. That is, $u(z(\mathbf{x}_t),\zeta(t)) = (g/2)(z(\mathbf{x}_t) - \zeta(t))^2$. The external force exerted by the trap on the molecule is $F_z(t) = (-)\partial u(z(\mathbf{x}_t), \zeta(t))/\partial z(\mathbf{x}_t) = -g(z(\mathbf{x}_t) - \zeta(t))$ and is obtained directly from the deflection (and intensity) of the trapping light beam. The force constant g is determined by a calibration, wherein the force, $F_{z(t)}$, and displacement, $z(\mathbf{x}_t) - \zeta(t)$, of the microsphere from the trap center are observed simultaneously. Once g is determined, a force measurement also provides $z(\mathbf{x}_t) - \zeta(t)$, which can be combined with $\zeta(t)$ to yield $z(\mathbf{x}_t)$. More precisely, the change $\mathrm{d}(z(\mathbf{x}_t) - \zeta(t))$ in a given time interval $\mathrm{d}t$ can be combined with $\mathrm{d}\zeta(t)$ (which is simultaneously measured by a light lever) to obtain $\mathrm{d}z(\mathbf{x}_t)$. The transferred work along the trajectory is then reckoned as

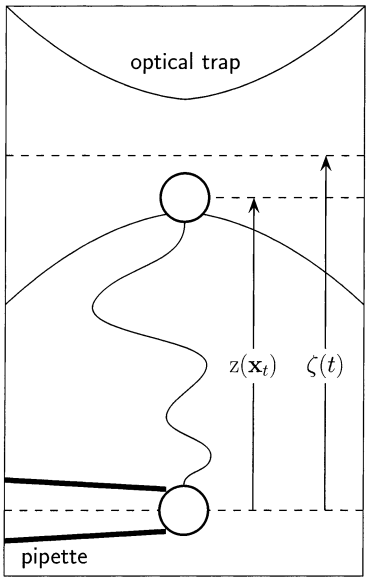

Figure 1. Schematic diagram of a single molecule-pulling experiment using an optical trap. The microsphere at the tail end is rigidly attached to a pipet, while the microsphere at the head end experiences the potential function of the optical trap. ζ(t) is the distance from the pipet to the trap center (position of null force), and $z(\mathbf{x}_t)$ is the molecular extension in the z direction.

$$w_\mathrm{T} = \int_{z_\mathrm{a}}^{z_\mathrm{b}} F_z(z(\mathbf{x}_{t'}),t')\, \mathrm{d}z(\mathbf{x}_{t'}) \tag{10}$$

where z_a and z_b are particular initial and final extensions.

An RNA molecule was ligated into the middle, between two DNA "handles". The entire chimeric molecule was then extended from some rather small extension through a region wherein the RNA molecule unfolds and beyond to still greater extension. At all pulling rates, the molecule was reported to behave reversibly, except in the region of the unfolding transition. The positions z_a and z_b were chosen to lie in the reversible, or equilibrium, regions on either side of the unfolding transition. Of course, the molecular extension could in principle pass through either z_a and z_b at multiple times. In the experiments, the fluctuating extensions along each trajectory in the reversible regions around z_a and z_b were smoothed or averaged so as to provide *unique* times at which z_a and z_b were traversed (Bustamante, C.; Smith, S. B. Personal communications). In that case, z_a and z_b correspond closely to the average extensions for the particular pipet−trap distances, $\zeta(t_\mathrm{a})$ and $\zeta(t_\mathrm{b})$, respectively, which presumably occur at practically the same times, $t_\mathrm{a} = 0$ and $t_\mathrm{b} = t$, in all trajectories. Because all trajectories considered begin at the same initial z_a and terminate at the same final z_b, the averaging over initial and final configurations of the system coordinates $\mathbf{x}$ is subject to those constraints and is therefore incomplete. This contrasts with the complete averaging in Jarzynski's equality (eq 2 above).

The Plan. The plan of the present work is as follows. First, some basic definitions and conditions needed for the general derivations are presented. Then the connection between Jarzynski's equality, potential function switching, and free-energy perturbation theory is discussed. After that, new equali-

ties involving $\exp(-\beta w_T)$ are derived and discussed. Then the average value of the reversible transferred work, $\langle w_T^{rev}\rangle$, is evaluated and analyzed, and its meaning is discussed. After that, equalities for $\langle\delta(z(\mathbf{x}_t) - z_f)\exp(-\beta(w_T(t) - u((\mathbf{x}_0),0)))\rangle$ and related densities are briefly derived via the same method and discussed. Next, the quantities $\langle\exp(-\beta w_T(t))\rangle_{z_a\to z_b}$ and $\langle\exp(-\beta w_A(t))\rangle_{z_a\to z_b}$ are defined, evaluated, and related to the respective reversible works, $\langle w_T^{rev}\rangle_{z_a\to z_b}$ and $\langle w_A^{rev}\rangle_{z_a\to z_b}$, for the single-molecule stretching experiments of Liphardt et al.[30] Then, the predictions are compared with the experimental results. Also in that same section, the newly derived relation for $\langle\exp(-\beta w_T(t))\rangle_{z_a\to z_b}$ is compared with the conjectured relation of Liphardt et al.[30] and the conditions required for validity of the latter are examined in detail. Finally, protocols for determining the free-energy profile of a single molecule in a stretching experiment, and for assessing the adequacy of the trajectory sampling, are suggested. A connection of the present relation for $\langle\exp(-\beta w_T(t))\rangle_{z_a\to z_b}$ to macroscopic thermodynamics is then discussed in detail.

Basic Definitions and Conditions. For the case of a system interacting with a much larger thermal reservoir, the extant proofs of Jarzynski's identity all invoke a balanced stochastic mapping assumption, according to which the system evolves in time in such a way that the canonical distribution for the prevailing instantaneous potential energy function is mapped into itself in any time step however short, regardless of the prior history of the interaction between the system and the thermal bath. Certainly, Brownian dynamics,[41-43] which is equivalent to overdamped simple Langevin dynamics, and Metropolis Monte Carlo (MC) protocols[1,2,7,44] evolve in such a manner.[39,40] The rather different protocol of *generalized* Langevin dynamics,[45] which incorporates some effective memory of the prior interaction of the system with the bath, does not appear to satisfy the stochastic mapping condition for arbitrarily short times (Schurr, J. M., unpublished "proof"). Here we focus on systems that evolve according to balanced stochastic dynamics, which is defined explicitly below.

The time from t_0 to t_N is divided into N elementary steps of equal duration dt. The times marking the *starts* of the different intervals are $t_0 = 0$, $t_1 = t_0 + dt$, ..., $t_j = t_{j-1} + dt$, ..., $t_N = t_{N-1} + dt$. The time intervals themselves are indexed according to their *ending* times, so the jth interval extends from t_{j-1} to t_j, for all $j = 1, ..., N$. The continuously evolving coordinates take the values $\mathbf{x}_j = \mathbf{x}_{t_j}$ at t_j. Any switching of the potential energy *function* is assumed to occur instantly (in a negligibly short time) *at fixed system coordinates*, $\mathbf{x}_j$, at the *starts* of one or more time intervals. The external potential function throughout the jth interval is that prevailing immediately *after* the potential switch at t_{j-1} and is denoted by $u_j(\mathbf{x}) = \lim_{0\leftarrow\epsilon} u(\mathbf{x},t_{j-1}+\epsilon)$. The *total* potential energy *function* is assumed to retain the same form, $V_j(\mathbf{x}) = V(\mathbf{x}) + u_j(\mathbf{x})$, throughout the jth interval from t_{j-1} to t_j for all $j = 1, ..., N$. In this notation, $u_0(\mathbf{x}) = u(\mathbf{x},t<0)$ is the external potential function prevailing at times *before* $t = 0$.

If $u_0(\mathbf{x})$ is nonvanishing, as is permitted here, it must be included in the canonical weighting of the initial coordinates. In that case, $V_0(\mathbf{x}) = V(\mathbf{x}) + u_0(\mathbf{x})$ and

$$Z_0^C = \int d\mathbf{x}\,\exp(-\beta(V(\mathbf{x}) + u_0(\mathbf{x}))) \tag{11}$$

The probability density that the system evolves (under $V_j(\mathbf{x})$) from $\mathbf{x}_{j-1}$ to $\mathbf{x}_j$ during the jth interval is $g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)$. The balanced stochastic mapping assumption is expressed as

$$\int d\mathbf{x}_{j-1}\, e^{-\beta V_j(\mathbf{x}_{j-1})}\, g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) = e^{-\beta V_j(\mathbf{x}_j)} \tag{12}$$

Connection of Jarzynski's Equality to Potential Function Switching and Free Energy Perturbation Theory. It is emphasized here that Jarzynski's equality pertains to the accumulated potential energy due to potential function switching, or time variation of $u(\mathbf{x},t)$, at fixed $\mathbf{x}$ rather than to the work transferred from the externally applied potential, $u(\mathbf{x},t)$, to the bare system. This is easily seen, by deriving eq 2 using a succession of free energy perturbation theory steps interleaved with stochastic evolution steps. The ratio of final to initial partition functions is written successively as

$$\begin{aligned}
Z_N^C/Z_0^C &\equiv (Z_0^C)^{-1}\int d\mathbf{x}_N\, e^{-\beta V_N(\mathbf{x}_N)}\\
&= (Z_0^C)^{-1}\int\int d\mathbf{x}_N d\mathbf{x}_{N-1}\, e^{-\beta V_N(\mathbf{x}_{N-1})}\, g_N(\mathbf{x}_{N-1}|\mathbf{x}_N)\\
&= (Z_0^C)^{-1}\int\int d\mathbf{x}_N d\mathbf{x}_{N-1}\, e^{-\beta V_{N-1}(\mathbf{x}_{N-1})}\{e^{-\beta(V_N(\mathbf{x}_{N-1}) - V_{N-1}(\mathbf{x}_{N-1}))}\\
&\qquad\qquad g_N(\mathbf{x}_{N-1}|\mathbf{x}_N)\}\\
&= (Z_0^C)^{-1}\int\ldots\int d\mathbf{x}_N\ldots d\mathbf{x}_0\, e^{-\beta V_0(\mathbf{x}_0)}\prod_{j=1}^{N}\{e^{-\beta(u_j(\mathbf{x}_{j-1}) - u_{j-1}(\mathbf{x}_{j-1}))}\\
&\qquad\qquad g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)\}\\
&= \langle\exp(-\beta\sum_{j=1}^{N}(u_j(\mathbf{x}_{j-1}) - u_{j-1}(\mathbf{x}_{j-1})))\rangle\\
&= \langle\exp(-\beta\sum_{j=1}^{N}(u(\mathbf{x}_{j-1},t_j) - u(\mathbf{x}_{j-1},t_{j-1})))\rangle\\
&= \langle e^{-\beta w_A(t)}\rangle
\end{aligned} \tag{13}$$

The second line of eq 13 is obtained from the first by using the balanced mapping condition (12). Specifically, the left-hand side of eq 12 with $j = N$ is substituted for $\exp(-\beta V_N(\mathbf{x}_N))$. The third line is obtained from the second by writing $\exp(-\beta V_N(\mathbf{x}_{N-1}))$ as $\exp(-\beta V_{N-1}(\mathbf{x}_{N-1}))\exp(-\beta V_N(\mathbf{x}_{N-1}) - V_{N-1}(\mathbf{x}_{N-1}))$, as is done in free energy perturbation theory. Beginning with the $\exp(-\beta V_{N-1}(\mathbf{x}_{N-1}))$ factor outside the braces in the third line, the same two steps are iterated in the same order. That is, $\exp(-\beta V_j(\mathbf{x}_j))$ with $j = N - 1$ is substituted by the balanced mapping relation, and the $\exp(-\beta V_j(\mathbf{x}_{j-1}))$ in the resulting expression is expressed as

$$\begin{aligned}
&\exp(-\beta V_{j-1}(\mathbf{x}_{j-1}))\exp(-\beta(V_j(\mathbf{x}_{j-1}) - V_{j-1}(\mathbf{x}_{j-1}))) =\\
&\qquad\exp(-\beta V_{j-1}(\mathbf{x}_{j-1}))\exp(-\beta(u_j(\mathbf{x}_{j-1}) - u_{j-1}(\mathbf{x}_{j-1})))
\end{aligned}$$

Successive iterations of these same two steps lead to the fourth line in eq 13, which can be recognized as an average of the exponential quantities in the fifth line over all possible canonically weighted initial conditions and all possible trajectories. In the limit $dt \to 0$ and $N \to \infty$, such that $N\,dt$ remains constant, the sum in the exponent of the sixth line becomes equal to $w_A(t)$ in eq 3 and the seventh line can be written as indicated in the sixth line. Of course, $Z_N^C/Z_0^C = Z_t^C/Z_0^C$, so eq 13 is recognized as Jarzynski's equality in eq 2. The method used to evaluate path integrals in eq 13 and elsewhere in this work is fundamentally similar to that employed by Jarzynski,[39] who proceeded in the opposite direction. Although the inverse perturbation theory steps, like that proceeding from the third to the second line in eq 13, were clearly displayed, their close connection to free energy perturbation theory was not mentioned.

The changes in potential energy function, $V_j(\mathbf{x}_j) - V_{j-1}(\mathbf{x}_j) = u_j(\mathbf{x}_j) - u_{j-1}(\mathbf{x}_j)$, at fixed $\mathbf{x}_j$ at any given t_j may be arbitrarily large. Indeed, the entire jump in external potential function may take place at any single t_j, $j = 0, ..., N - 1$. Jarzynski's equality can be regarded as a multistep dynamical free-energy perturbation theory for any system that evolves entirely according to

the balanced mapping condition (12). The essential point is that it concerns *changes* in potential energy function at instantaneously *fixed* system coordinates and takes *no* account of work *transferred* from the external potential, $u(\mathbf{x},t)$, to the bare system, as that system moves in response to the external potential. The balanced mapping condition must be obeyed for *arbitrarily small* times after the potential function is switched, but the actual extent of displacement of the system is without consequence for validity of Jarzynski's equality. In fact, the extent of motion could be reduced to zero, for example, by raising the viscosity to an infinitely large value, and Jarzynski's equality would be unaffected. However, in that case, the sampling of initial conditions would have to include all of the states that are significantly weighted by the final potential as well as those that are significantly weighted by the initial potential.

In the case of MC evolution, the entire process is divided into N equivalent steps but it is not necessary to associate a time duration with each. This again illustrates that Jarzynski's equality concerns a multistep potential function switching process that in essence is independent of any *temporal* evolution of the bare system.

An Equality for the Transferred Work $w_T(t)$. During the jth time interval from t_{j-1} to t_j, the work transferred from the external potential to the bare system is due to displacement of the system from $\mathbf{x}_{t_{j-1}}$ to $\mathbf{x}_{t_j}$ in the presence of that external potential and is given by the last expression in eq 4. The same abbreviations and conventions of the preceding section apply here. The quantity $w_T(t)$ in eq 4 can be rewritten in discrete notation as

$$w_T(t) = (-)\sum_{j=1}^{N}(u_j(\mathbf{x}_j) - u_j(\mathbf{x}_{j-1})) \tag{14}$$

We consider first the general case, when $u_0(\mathbf{x})$ is not required to vanish and then admit the special case $u_0(\mathbf{x}) = 0$ at the end. The present method allows evaluation of $\langle\exp(-\beta(w_T(t) + u_0(\mathbf{x}_N) - u_0(\mathbf{x}_0)))\rangle$, which reduces to $\langle\exp(-\beta w_T(t))\rangle$, when $u_0(\mathbf{x}) = 0$. The average over all canonically distributed initial conditions and all trajectories is

$$\langle e^{-\beta(w_T(t)+u_0(\mathbf{x}_N)-u_0(\mathbf{x}_0))}\rangle = (Z_0^C)^{-1}\int\ldots\int d\mathbf{x}_N \ldots d\mathbf{x}_0\, e^{-\beta(V(\mathbf{x}_0)+u_0(\mathbf{x}_0))} e^{\beta u_0(\mathbf{x}_0)}\{\prod_{j=1}^{N} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) e^{\beta(u_j(\mathbf{x}_j)-u_j(\mathbf{x}_{j-1}))}\} e^{-\beta u_0(\mathbf{x}_N)} \tag{15}$$

where Z_0^C is given by eq 11. The $u_0(\mathbf{x}_0)$ terms in the exponent cancel. Use of the balanced mapping condition for the prevailing potential $V_1(\mathbf{x}_0) = V(\mathbf{x}_0) + u_1(\mathbf{x}_0)$ in the $d\mathbf{x}_0$ integral gives

$$\int d\mathbf{x}_0\, e^{-\beta V(\mathbf{x}_0)}\, g_1(\mathbf{x}_0|\mathbf{x}_1) e^{\beta(u_1(\mathbf{x}_1)-u_1(\mathbf{x}_0))} = e^{-\beta V(\mathbf{x}_1)} \tag{16}$$

This result is incorporated into the $d\mathbf{x}_1$ integral. After applying the balanced mapping condition for the potential function $V(\mathbf{x}_1) + u_2(\mathbf{x}_1)$ and canceling the $\exp(-\beta(u_2(\mathbf{x}_2)))$ and $\exp(\beta u_2(\mathbf{x}_2))$ factors in the integrand, there results $\exp(-\beta(V(\mathbf{x}_2)))$. This process is iterated in a sequential manner through the $d\mathbf{x}_{N-1}$ integral to obtain $\exp(-\beta(V\mathbf{x}_N) + u_0(\mathbf{x}_N)))$ which is the entire integrand of the remaining $d\mathbf{x}_N$ integral, hence

$$\langle e^{-\beta(w_T(t)+u_0(\mathbf{x}_N)-u_0(\mathbf{x}_0))}\rangle = (Z_0^C)^{-1}\int d\mathbf{x}_N\, e^{-\beta(V(\mathbf{x}_N)+u_0(\mathbf{x}_N))} = 1.0 \tag{17}$$

When $u_0(\mathbf{x}) = 0$, eq 17 becomes

$$\langle\exp(-\beta^{w_T(t)})\rangle = 1.0 \tag{18}$$

Equation 18 was independently derived for a one-dimensional system via the Feynman−Kac theorem by H. Qian (personal communication). Equation 17 can also be derived from Jarzynski's equality (2) and eq 5 by considering a particular "cyclic" variation of the potential function, so $Z_t^C/Z_0^C = 1.0$. Specifically, if the potential function is suddenly switched from $u_N(\mathbf{x})$ to $u_0(\mathbf{x})$ in an additional time step of infinitesimal *duration at the end of the original trajectory*, then the system configuration will remain unchanged at $\mathbf{x}_N$, and $w_T(t)$ will remain unchanged at its original value, but eq 2 will become eq 18. This same point was noted by an anonymous reviewer.

Equations 17 and 18 also apply to the case wherein the system is first isolated from the heat bath and then propagated adiabatically according to classical mechanics. The argument is virtually identical to that presented above except that the "coordinate" list $\mathbf{x}$ is doubled to include the momenta conjugate to the coordinates and the intrinsic potential $V(\mathbf{x})$ is replaced by the intrinsic Hamiltonian $\mathbf{H}_0(\mathbf{x}) = K(\mathbf{x}) + V(\mathbf{x})$, where $K(\mathbf{x})$ is the intrinsic kinetic energy function.

The somewhat counterintuitive result in eq 18 is critically dependent on (1) averaging over all possible starting configurations of the system, which are weighted according to the canonical distribution for the initial potential $V(\mathbf{x})$, and (2) averaging over all possible terminal configurations of the trajectories. If either of these averages is omitted, then substantially different results are obtained, as will be demonstrated below. The unexpected result in eq 18 is attributable primarily to a small fraction of the trajectories, which due to Brownian fluctuations move in a direction opposite to the external force(s) and thereby generate negative values of $w_T(t)$.

Despite the fact that $\langle\exp(-\beta w_T(t))\rangle = 1.0$ when $u_0(\mathbf{x}) = 0$, the average value of $w_T(t)$ is generally positive, does not vanish except at $t = 0$, and is time dependent. Moreover, in most cases, the quantity $w_T(t)$ is not entirely dissipative work, since some of the transferred work goes into system displacements that increase the value of the intrinsic potential energy. These assertions are addressed in the next section.

The difference between eq 17 on one hand and eq 13 on the other illustrates that considerable care must be exercised in the choice of work in such nonequilibrium work equalities. It is also conceivable that eq 17 might provide a useful test for the adequacy of trajectory sampling in some cases.

Two additional equalities can be evaluated by precisely the same method

$$\langle\exp(-\beta(w_T(t) + (u_N(\mathbf{x}_N) - u_0(\mathbf{x}_0))))\rangle = (Z_0^C)^{-1}\int\ldots\int d\mathbf{x}_N \ldots d\mathbf{x}_0\, e^{-\beta(V(\mathbf{x})+u_0(\mathbf{x}_0))} e^{\beta u_0(\mathbf{x}_0)} \prod_{j=1}^{N} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)\, e^{\beta(u_j(\mathbf{x}_j)-u_j(\mathbf{x}_{j-1}))}\, e^{-\beta u_N(\mathbf{x}_N)} = (Z_0^C)^{-1}\int d\mathbf{x}_N\, e^{-\beta(V(\mathbf{x}_N)+u_N(\mathbf{x}_N))} = Z_N^C/Z_0^C = e^{-\beta(A_t^C - A_0^C)} \tag{19}$$

Equation 19 is an alternative form of Jarzynski's equality, as expected from eq 5.

When the $u_N(\mathbf{x}_N)$ term is omitted from the exponents on both sides of eq 19 but the $u_0(\mathbf{x}_0)$ term is retained, one obtains

$$\langle\exp(-\beta(w_T(t) - u_0(\mathbf{x}_0)))\rangle = Z_0^{in}/Z_0^C = e^{-\beta(A_0^{in} - A_0^C)} \tag{20}$$

where $A_0^{in} = -kT \ln Z_0^{in}$, $A_0^C = -kT \ln Z_0^C$, and Z_0^{in} and Z_0^C are

given in eqs 8 and 11, respectively. Although eq 20 is reckoned for a system that begins at potential function $V(\mathbf{x}) + u_0(\mathbf{x})$ at $t = 0$ and evolves to $V(\mathbf{x}) + u_N(\mathbf{x})$ at time t, the final result is entirely independent of either $u_N(\mathbf{x}) = u(\mathbf{x},t)$ or t. Thus, even if the external potential function is held fixed at $u_0(\mathbf{x})$ from $t = 0$ to t, or instead is reduced to 0 between $t = 0$ and t, this result is unchanged. Moreover, the trajectories could be of arbitrarily short duration. When the external potential is held fixed at $u_0(\mathbf{x})$ from $t = 0$ to t and the duration of the trajectory is limited to a single time step, eq 20 is completely equivalent to the free-energy perturbation theory with reference potential, $V(\mathbf{x}) + u_0(\mathbf{x})$ and perturbation potential $\delta u(\mathbf{x}) = -u(\mathbf{x}_0)$. If the external potential is reduced from $u_0(\mathbf{x})$ to 0 between $t = 0$ and t, eq 20 simply corresponds to Jarzynski's equality for that particular case.

In summary, eqs 19 and 20 provide alternative expressions of known equalities. They might prove useful in certain special cases, where the external forces are more directly available than the external potential energy function, which nevertheless must be known at the initial and final times for eq 19 and at the initial time for eq 20.

Evaluation and Significance of $\langle w_T^{rev}\rangle$. In the limit that the external potential is switched sufficiently slowly, the conditional probabilities become the appropriate canonical distributions

$$g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) = \exp(-\beta(V(\mathbf{x}_j) + u_j(\mathbf{x}_j)))/Z_j^C \qquad (21)$$

where

$$Z_j^C \equiv \int d\mathbf{x} \exp(-\beta(V(\mathbf{x}) + u_j(\mathbf{x}))) \qquad (22)$$

In this case

$$\langle w_T^{rev}\rangle = \int ... \int d\mathbf{x}_N ... d\mathbf{x}_0 \prod_{j=0}^{N} (e^{-\beta(V(\mathbf{x}_j)+u_j(\mathbf{x}_j))}/Z_j^C) \{(-)\sum_{k=1}^{N}(u_k(\mathbf{x}_k) - u_k(\mathbf{x}_{k-1}))\} = + \langle u_1(x_0)\rangle_0 + \sum_{k=1}^{N-1}\langle u_{k+1}(\mathbf{x}_k) - u_k(\mathbf{x}_k)\rangle_k - \langle u_N(\mathbf{x}_N)\rangle_N \qquad (23)$$

wherein

$$\langle u_l(\mathbf{x}_j)\rangle_j \equiv (Z_j^C)^{-1} \int d\mathbf{x}_j \, e^{-\beta(V(\mathbf{x}_j)+u_j(\mathbf{x}_j))} u_l(\mathbf{x}_j) \qquad (24)$$

is the average of $u_l(\mathbf{x})$ over the canonical distribution for $V_j(\mathbf{x}) = V(\mathbf{x}) + u_j(\mathbf{x})$. The indexed system coordinates are integrated out in the averaging and are included on the left side of eq 24 only for clarity. According to thermodynamics, $\langle w_T^{rev}\rangle$ is the change in Helmholtz free energy of the *bare* system, excluding the external potential. It can be compared with the corresponding change in free energy of the composite system, which is reckoned by a reversible work calculation in the appropriate notation. After setting $V_{j+1}(\mathbf{x}) = V(\mathbf{x}) + u_{j+1}(\mathbf{x}) = V(\mathbf{x}) + u_j(\mathbf{x}) + \delta u_j(\mathbf{x})$, where $\delta u_j(\mathbf{x}) = u_{j+1}(\mathbf{x}) - u_j(\mathbf{x})$, standard free energy perturbation theory gives

$$A_{j+1}^C - A_j^C = -\beta^{-1}\ln(Z_{j+1}^C/Z_j^C) = -\beta^{-1}\ln\langle\exp(-\beta\delta u_j(\mathbf{x}_j))\rangle_j = + \langle\delta u_j(\mathbf{x})\rangle_j = \langle u_{j+1}(\mathbf{x}) - u_j(\mathbf{x})\rangle_j \qquad (25)$$

where the angular brackets with subscripts j denote averages over the canonical distribution for $V_j(\mathbf{x}) = V(\mathbf{x}) + u_j(\mathbf{x})$. In proceeding from the second line of eq 25 to the third, it is assumed that the change in potential is sufficiently small (infinitesimal) that the exponential can be expanded to first order. Now, using eq 25

$$A_N^C - A_0^C = -\beta^{-1}\ln(Z_N^C/Z_0^C) = -\beta^{-1}\ln(\prod_{j=0}^{N-1}(Z_{j+1}^C/Z_j^C)) = \sum_{j=0}^{N-1}\langle u_{j+1}(\mathbf{x}_j) - u_j(\mathbf{x}_j)\rangle_j \qquad (26)$$

Comparison of eq 26 with 23 gives

$$\langle w_T^{rev}\rangle = A_N^C - A_0^C - (\langle u_N(\mathbf{x}_N)\rangle_N - \langle u_0(\mathbf{x}_0)\rangle_0) \qquad (27)$$

$\langle w_T^{rev}\rangle$ is the Helmholtz free energy change of the *bare* system, which excludes the external potential, and is equal to the Helmhotz free energy change of the composite system, which includes the external potential, minus the change in average value of the external potential. This result also is expected, because $\langle w_T^{rev}\rangle = \langle w_A^{rev}\rangle - (\langle u_N(\mathbf{x}_N)\rangle - \langle u_0(\mathbf{x}_0)\rangle)$ and $\langle w_A^{rev}\rangle = A_N^C - A_0^C$.

It is now assumed that $u(\mathbf{x},0) = 0 = u_0(\mathbf{x})$. From eq 18 and the mathematical identity, $\langle\exp(y)\rangle \geq \langle\exp(\langle y\rangle)\rangle$, it follows that $\langle w_T(t)\rangle \geq 0$; hence $\langle w_T^{rev}\rangle \geq 0$. In this case, then, $\langle w_T^{rev}\rangle$ is *always* positive, except when there is no change in potential function, in which case $\langle w_T^{rev}\rangle = 0$. This is expected, because $\langle w_T^{rev}\rangle$ is the minimum average work required to "deform" the equilibrium distribution of configurations of the bare system for the potential function $V(\mathbf{x})$ to that appropriate for the potential function $V(\mathbf{x}) + u_N(\mathbf{x})$, while excluding any contribution from changes in the external potential energy itself. In other words, $\langle w_T^{rev}\rangle$ is the work required to achieve a particular *nonequilibrium* macrostate of the *bare* system, which corresponds to its *equilibrium macrostate in the composite (perturbed) system* with external potential function $u_N(\mathbf{x})$. These remarks are clarified in the following paragraph.

A *macrostate* of the bare system is defined here by its particular *distribution* of configurations, $f(\mathbf{x})$. At equilibrium in the unperturbed bare system, $f(\mathbf{x}) = \exp(-\beta(V(\mathbf{x})))/Z_0^{in}$. Any other distribution corresponds to a fluctuated macrostate of the bare system. Hence, the equilibrium distribution of the perturbed system in the presence of the final potential, $V(\mathbf{x}) + u_N(\mathbf{x})$, is a *nonequilibrium* distribution of the bare system. Consider a new energy-shifted system (sh), in which the potential energy function is that of the perturbed composite system, $V(\mathbf{x}) + u_N(\mathbf{x})$, but the zero of energy is shifted to $E_0 = \langle u_N(\mathbf{x})\rangle$. At equilibrium, this shifted system has the distribution of configurations, $f_{sh}(\mathbf{x}) = \exp(-\beta(V(\mathbf{x}) + u_N(\mathbf{x}) - E_0)]/Z_{sh}$, where $Z_{sh} = \int d\mathbf{x} \exp(-\beta(V(\mathbf{x}) + u_N(\mathbf{x}) - E_0))$. This distribution is actually identical to that of the composite system; however, this *shifted* system has none of the *average* external potential energy of the composite system. This shifted system has precisely the distribution of configurations and vanishing average external potential energy of the fluctuated macrostate of the bare system that is under discussion. A key feature of this fluctuated macrostate is that its configuration energies are reckoned relative to E_0 rather than to $E = 0$. The ratio of the probability (P_{fl}) of the fluctuated macrostate to that (P_0) of the equilibrium macrostate of the bare system is the ratio of the sum over configurations of the unnormalized configuration probabilities, taking account of the shift in zero of energy of the fluctuated state

$$P_{\mathrm{fl}}/P_0 = \frac{\int \mathrm{d}\mathbf{x}\, \mathrm{e}^{-\beta(V(\mathbf{x})+u_N(\mathbf{x})-E_0)}}{\int \mathrm{d}\mathbf{x}\, \mathrm{e}^{-\beta V(\mathbf{x})}} = \frac{Z_N \mathrm{e}^{-\beta E_0}}{Z_0} = \mathrm{e}^{-\beta(A_N - A_0 - \langle u_N(\mathbf{x})\rangle)} = \mathrm{e}^{-\beta\langle w_{\mathrm{T}}^{\mathrm{rev}}\rangle} \quad (28)$$

Thus, $\langle w_{\mathrm{T}}^{\mathrm{rev}}\rangle$ governs the relative probability of the fluctuated *macrostate*. Because $\langle w_{\mathrm{T}}^{\mathrm{rev}}\rangle \geq 0$, one also has $P_{\mathrm{fl}}/P_0 \leq 1.0$, as expected.

Examples Involving Brownian Particles. Equation 18 was initially discovered while evaluating $\langle \exp(-\beta w_{\mathrm{T}}(t))\rangle$ for specific examples involving Brownian particles in various time-dependent external potentials. Simple overdamped Langevin equations, or the equivalent Brownian dynamics relations, were solved analytically for (1) a free particle subject to a time-dependent uniform external force, (2) a Brownian particle in a harmonic potential well with a uniformly translating equilibrium position, and (3) a Brownian particle in a harmonic potential well subjected to an additional time-dependent uniform force. In all three cases, eq 18 was found to hold exactly. These analyses will be published elsewhere along with numerical investigations of the convergence for different temporal variations of the applied forces.

Evaluation of $\langle \delta(z_{\mathrm{f}} - z(\mathbf{x}_t))\, \exp[-\beta(w_{\mathrm{T}}(t) - u_0(\mathbf{x}_0))]\rangle$. The case wherein the external potential depends on the system coordinates via a single extensional parameter, $z(\mathbf{x})$, is now considered explicitly. Throughout this discussion, $z(\mathbf{x})$ can be regarded as the extension, or end−end distance, in the z direction. The potential function becomes $u(z(\mathbf{x}_t),t) = u(z_t,t)$, where $z_t \equiv z(\mathbf{x}_t)$, and

$$w_{\mathrm{T}}(t) = \int_{z_0}^{z_t} F(z_{t'},t')\, \mathrm{d}z_{t'} = \int (-) \frac{\partial u(z_{t'},t')}{\partial z_{t'}} \frac{\partial z_{t'}}{\partial \mathbf{x}_{t'}} \cdot \mathrm{d}\mathbf{x}_{t'} = (-) \int (u(z(\mathbf{x}_{t'}+\mathrm{d}\mathbf{x}_{t'}),t') - u(z(\mathbf{x}_{t'}),t')) \quad (29)$$

A Taylor series for $u(z(\mathbf{x}_t'+\mathrm{d}\mathbf{x}_{t'}),t')$ in the final expression leads to the penultimate expression in eq 29.

The time is subdivided into N equal intervals indexed exactly as before. The abbreviated forms, $\mathbf{x}_j = \mathbf{x}_{t_j}$, $z_j = z(\mathbf{x}_j)$, and $u_j(\mathbf{x}) = u_j(z(\mathbf{x})) = \lim_{\epsilon \leftarrow 0} u(z(\mathbf{x}),t_{j-1}+\epsilon)$ are employed, and $w_{\mathrm{T}}(t)$ in eq 29 is again given by eq 14.

Our objective is to evaluate $\langle \exp(-\beta(w_{\mathrm{T}}(t) - u_0(\mathbf{x}_0)))\delta(z(\mathbf{x}_N) - z_{\mathrm{f}})\rangle$ for the case when $u_0(\mathbf{x})$ is not required to vanish. For the special case when $u_0(\mathbf{x}) = 0$, this becomes $\langle \exp(-\beta w_{\mathrm{T}}(t))\delta(z(\mathbf{x}_N) - z_{\mathrm{f}})\rangle$. The delta function admits contributions only from trajectories that terminate at a given final extension z_{f} and changes the units to density in the one-dimensional space of final extensions, $z(\mathbf{x}_N)$. Because the delta function $\delta(z(\mathbf{x}_t) - z_{\mathrm{f}}) = \delta(z(\mathbf{x}_N) - z_{\mathrm{f}})$ affects only the last coordinates integrated (i.e., $\mathbf{x}_N$), the preceding $N-1$ integrals are completely unaffected, and the evaluation proceeds exactly as for eq 17 up until the final integration, which then yields eq 6 above. This demonstrates that eq 6, which was originally derived by Hummer and Szabo by using the Feynman−Kac theorem and eq 5, can also be derived by direct evaluation of the path integral. A key point regarding eq 6 with $u_0(\mathbf{x}_0) = 0$ is that restricting the trajectories to those that terminate at z_{f} at time t effectively excludes from the averaging most or all of those trajectories with transferred works of opposite sign to $A_0^{\mathrm{in}}(z_{\mathrm{f}})$, which are required to obtain eq 18.

After multiplying both sides of eq 6 by $\mathrm{d}z_{\mathrm{f}}$ and integrating over z_{f}, one finds

$$\int \mathrm{d}z_{\mathrm{f}} \langle \delta(z(\mathbf{x}_N) - z_{\mathrm{f}}) \exp(-\beta(w_{\mathrm{T}}(t) - u_0(\mathbf{x}_0)))\rangle = \langle \exp(-\beta(w_{\mathrm{T}}(t) - u_0(\mathbf{x}_0)))\rangle = Z_0^{\mathrm{in}}/Z_0^{\mathrm{C}} \quad (30)$$

where Z_0^{in} and Z_0^{C} are given in eqs 8 and 11, respectively. The right-hand side of eq 30 contains *no* information pertaining to either Z_N^{C} or $Z_0^{\mathrm{in}}(z_{\mathrm{f}})$ but instead contains only Z_0^{in} for the bare system and Z_0^{C} for the *initial* composite system and takes the value 1.0, when $u_0(\mathbf{x}_0) = 0$. This illustrates how averaging $\langle \exp(-\beta w_{\mathrm{T}}(t) - u_0(\mathbf{x}_0))\delta(z(\mathbf{x}_N) - z_{\mathrm{f}})\rangle$ over all possible z_{f} values effectively removes all information about the *final* states of either the composite system or the bare system.

An additional equality can be derived by multiplying both sides of eq 6 by $\exp(-\beta u_N(z_{\mathrm{f}}))$ and recognizing that this constant factor may be incorporated inside the averaging integral on the left-hand side as $\exp[-\beta u_N(z(\mathbf{x}_N))]$ because the delta function constrains $z(\mathbf{x}_N)$ to z_f. The resulting expression can be written explicitly in terms of the time as

$$\langle \delta(z(\mathbf{x}_t) - z_{\mathrm{f}}) \exp(-\beta(w_{\mathrm{T}}(t) + u(z(\mathbf{x}_t),t) - u(z(\mathbf{x}_0),0)))\rangle = \langle \delta(z(\mathbf{x}_t) - z_{\mathrm{f}}) \exp(-\beta w_{\mathrm{A}}(t))\rangle = \exp(-\beta(A_0^{\mathrm{in}}(z_{\mathrm{f}}) + u(z_{\mathrm{f}},t))) \quad (31)$$

Equation 5 was used to go from the first line to the second. This relation is another rearrangement of the result of Hummer and Szabo.[36] Whenever the work transferred from the surroundings to the composite system, $w_{\mathrm{A}}(t)$, is more readily available than the work transferred from the external potential to the bare system, $w_{\mathrm{T}}(t)$, eq 31 might be more useful than eq 6.

Of the presently available nonequilibrium work equalities, eqs 6 and 31 are the most pertinent for experimental determination of the free-energy profile of the bare system (i.e., the molecule being stretched). However, they do not apply directly to the experiments and data of Liphardt et al.,[30] because of the different way in which the initial state averaging was done in that work.

Evaluation of $\langle \exp[-\beta w_{\mathrm{T}}(t)]\rangle_{\mathbf{z_a}\rightarrow\mathbf{z_b}}$ for Single-Molecule Stretching Experiments. The objective now is to derive expressions for analyzing single molecule stretching experiments, like that of Liphardt et al.[30] The bare molecular system is taken to include the microspheres, if any, that are attached at either end. The tail end of the molecular system is rigidly attached to a stationary anchor, such as a pipet, and the head end is assumed to experience a force due to an external potential, which could arise from an optical trap, a cantilever "spring", a magnetic or electric field, or field gradient. The instantaneous external potential energy, $u(z(\mathbf{x}_t),\zeta(t))$, is assumed to depend only on the instantaneous extension, $z(\mathbf{x}_t)$, of the molecular system in the z direction and on the distance $\zeta(t)$ (in the z direction) from the rigidly fixed tail position of the molecule to a reference position in the potential "field" experienced by the head end of the molecule. In the case of an optical trap, $\zeta(t)$ is the distance from the fixed tail position to the center of the trap, where the head microsphere would experience a null force due to the trap (see Figure 1). In the case of a cantilever "spring", $\zeta(t)$ is the distance from the tail position to the point where the head of the molecule would experience a null force due to the spring. In the case of a uniform electric field or magnetic field gradient, there is no position of null force accessible to the "bead" at the head end of the molecule, so some other value of the force must be used to define the reference position and $\zeta(t)$. Time is discretized exactly as before, so $\mathbf{x}_j \equiv \mathbf{x}_{t_j}$, $z(\mathbf{x}_j) \equiv z(\mathbf{x}_{t_j})$, and $\zeta_j \equiv \zeta(t_j)$ and the external potential prevailing throughout the jth interval from t_{j-1} to t_j is $u_j(\mathbf{x}) = u_j(z(\mathbf{x})) \equiv \lim_{0\leftarrow\epsilon} u(z(\mathbf{x}),\zeta(t_{j-1}+\epsilon))$

$= u(z(\mathbf{x}),\zeta_{j-1})$. The force exerted on the configuration $\mathbf{x}_j$ by the external potential during the jth interval is in the z direction and has magnitude $F_{z_j} = (-)\partial u_j(z(\mathbf{x}_j))/\partial z(\mathbf{x}_j) = (-)\partial u(z(\mathbf{x}_j),\zeta_{j-1})/\partial z(\mathbf{x}_j)$. The external potential changes by discontinuous jumps in $\zeta(t)$ at times t_j. ζ then remains constant at the value $\zeta_{j-1} = \zeta(t_{j-1})$ throughout the jth interval. The transferred work can be written as

$$w_T(t) \equiv (-)\sum_{j=1}^{N}(u_j(\mathbf{x}_j) - u_j(\mathbf{x}_{j-1})) = \sum_{j=1}^{N} F_{z_j}\, dz(\mathbf{x}_j) \quad (32)$$

where $dz(\mathbf{x}_j) = z(\mathbf{x}_j) - z(\mathbf{x}_{j-1})$ is the net change in $z(\mathbf{x})$ during the jth time interval. Equation 32 is simply a discretized version of eq 10. The initial extension is assumed to take a particular value z_a, and only those trajectories that terminate at a particular final extension, z_b, are considered.

It is assumed that, at all experimental pulling rates, $\partial\zeta(t)/\partial t$, the system behaves reversibly in the vicinity of z_a and again in the vicinity of z_b, although it may exhibit irreversible behavior between those points. Under these conditions, one can select z_a to be the average value of $z(\mathbf{x}_0)$ at $t = 0$, when $\zeta(0) = \zeta_0$, and z_b to be the average value of $z(\mathbf{x}_t)$, when $\zeta(t) = \zeta_N$. Because of the reversible behavior near z_a and z_b, smoothing of the force vs extension trajectories in those regions is required to ensure that all smoothed trajectories pass through z_a only once and at a time close to $t = 0$, when $\zeta(0) = \zeta_0$, and likewise that z_b is traversed only once and at a time close to t, when $\zeta(t) = \zeta_N$. Then $u_0(\mathbf{x}_0) = u(z_a,\zeta_0)$ and $u_N(\mathbf{x}_N) = u(z_b,\zeta_N)$. The experiments necessarily admit trajectories starting in some small range dz_a containing z_a and terminate in some small range dz_b containing z_b. The average of $\exp(-\beta w_T(t))$ is taken over all of these selected trajectories. This average value should be independent of the size of dz_a and dz_b, provided those line elements are sufficiently small, and should be unitless. The appropriate quantity is

$$\langle e^{-\beta w_T(t)}\rangle_{z_a\to z_b} = \frac{\langle e^{-\beta w_T(t)}\delta(z(\mathbf{x}_0) - z_a)\delta(z(\mathbf{x}_N) - z_b)\rangle}{\langle \delta(z(\mathbf{x}_0) - z_a)\delta(z(\mathbf{x}_N) - z_b)\rangle} \quad (33)$$

The angular brackets again denote averages of the indicated quantities over all trajectories, but the trajectories differ from those considered previously in the sense that the initial steps are performed reversibly, as are the final steps. That is, full equilibrium is reached during the initial step and again during the final step of the trajectory, though not necessarily on all the intermediate steps. The delta functions enforce the constraints on the initial and final values of the extension. The numerator is an average density in the two-dimensional space of $z(\mathbf{x}_0)$ and $z(\mathbf{x}_N)$. The denominator is the appropriate *normalization density* in the same space for the trajectories considered. The same constraints that appear in the averaging integral in the numerator must also appear in the normalization integral in the denominator, and the initial and final steps are also reversible. A characteristic feature of the reversible initial and final stages of the experiments is that all "memory" of the starting extension z_a is lost before $\zeta(t)$ differs more than infinitesimally from $\zeta(0)$, and likewise no "foreknowledge" of the final extension z_b is acquired until $\zeta(t)$ is infinitesimally close to ζ_N. The actual time for such equilibration processes is not known and, if dt were sufficiently small, would consist of many steps. Here we assume that such equilibration takes place in a single step of unspecified time duration, $t_1 - t_0$ or $t_N - t_{N-1}$, such that $\zeta_1 = \zeta(t_1) \cong \zeta(0) = \zeta_0$ and $\zeta_{N-1} = \zeta(t_{N-1}) \cong \zeta(t_N) = \zeta_N$. These relations will frequently be invoked in the derivations presented in this section.

The numerator in eq 33 is developed as follows

$$\begin{aligned}
&\langle e^{-\beta w_T(t)}\delta(z(\mathbf{x}_0) - z_a)\delta(z(\mathbf{x}_t) - z_b)\rangle = \\
&\quad (Z_0^C)^{-1}\int\!\!\ldots\!\int d\mathbf{x}_N\ldots d\mathbf{x}_0\, e^{-\beta(V(\mathbf{x}_0)+u_0(\mathbf{x}_0))}\delta(z(\mathbf{x}_0) - z_a) \\
&\quad \frac{e^{-\beta(V(\mathbf{x}_1)+u_1(\mathbf{x}_1))}}{Z_1^C}\, e^{\beta(u_1(\mathbf{x}_1)-u_1(\mathbf{x}_0))}\{\prod_{j=2}^{N} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) \\
&\quad e^{\beta(u_j(\mathbf{x}_j)-u_j(\mathbf{x}_{j-1}))}\}\delta(z(\mathbf{x}_N) - z_b) = \\
&\quad (Z_0^C Z_1^C)^{-1}\, Z_0^{in}(z_a)\, e^{-\beta(u(z_a,\zeta_0)+u(z_a,\zeta_1))} \\
&\int\!\!\ldots\!\int d\mathbf{x}_N\ldots d\mathbf{x}_1\, e^{-\beta(V(\mathbf{x}_1)+u_2(\mathbf{x}_1))} g_2(\mathbf{x}_1|\mathbf{x}_2)\, e^{\beta u_2(\mathbf{x}_2)} \\
&(\prod_{j=3}^{N} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) e^{\beta(u_j(\mathbf{x}_j)-u_j(\mathbf{x}_{j-1}))})\delta(z(\mathbf{x}_N) - z_b) = \\
&\quad (Z_0^C Z_1^C)^{-1} Z_0^{in}(z_a) e^{-\beta(u(z_a,\zeta_0)+u(z_a,\zeta_1))} \\
&\int d\mathbf{x}_N\, e^{-\beta V(\mathbf{x}_N)}\delta(z(\mathbf{x}_N) - z_b) = \frac{Z_0^{in}(z_a)Z_0^{in}(z_b)}{Z_0^C Z_1^C}\, e^{-\beta(u(z_a,\zeta_0)+u(z_a,\zeta_1))}
\end{aligned} \quad (34)$$

In the first line on the right-hand side of eq 34, the first step is assumed to be reversible, so $g_1(\mathbf{x}_0|\mathbf{x}_1)$ is given by eq 21 and Z_j^C is given by eq 22. The third line follows from the second by repeated application of the detailed balance condition (eq 12).

The denominator in eq 33 is evaluated as follows

$$\begin{aligned}
&\langle \delta(z(\mathbf{x}_0) - z_a)\delta(z(\mathbf{x}_N) - z_b)\rangle = \\
&\quad (Z_0^C)^{-1}\int\!\!\ldots\!\int d\mathbf{x}_N\ldots\, d\mathbf{x}_0\, e^{-\beta(V(\mathbf{x}_0)+u_0(\mathbf{x}_0))}\delta(z(\mathbf{x}_0) - z_a) \\
&\quad \frac{e^{-\beta(V(\mathbf{x}_1)+u_1(\mathbf{x}_1))}}{Z_1^C}\{\prod_{j=2}^{N-1} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)\} \\
&\quad \frac{e^{-\beta(V(\mathbf{x}_N)+u_N(\mathbf{x}_N))}}{Z_N^C}\,\delta(z(\mathbf{x}_N) - z_b) = \\
&\quad (Z_0^C Z_1^C)^{-1}\, Z_0^{in}(z_a)\, e^{-\beta u(z_a,\zeta_0)} \\
&\int\!\!\ldots\!\int d\mathbf{x}_N\ldots\, d\mathbf{x}_1\, e^{-\beta(V(\mathbf{x}_1)+u_1(\mathbf{x}_1))}\, g_2(\mathbf{x}_1|\mathbf{x}_2) g_3(\mathbf{x}_2|\mathbf{x}_3)\ldots \\
&\quad g_{N-1}(\mathbf{x}_{N-2}|\mathbf{x}_{N-1})\frac{e^{-\beta(V(\mathbf{x}_N)+u_N(\mathbf{x}_N))}}{Z_N^C}\,\delta(z(\mathbf{x}_N) - z_b)
\end{aligned} \quad (35)$$

Equation 21 has been used for both $g_1(\mathbf{x}_0|\mathbf{x}_1)$ and $g_N(\mathbf{x}_{N-1}|\mathbf{x}_N)$. Use is now made of the normalization relations for the transition probability densities

$$\int d\mathbf{x}_j\, g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)\, g_{j+1}(\mathbf{x}_j|\mathbf{x}_{j+1}) = g_{j\to j+1}(\mathbf{x}_{j-1}|\mathbf{x}_{j+1}) \quad (36)$$

$$\int d\mathbf{x}_j\, g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) = 1.0 = \int d\mathbf{x}_{j-1}\, g_j(\mathbf{x}_{j-1}|\mathbf{x}_j) \quad (37)$$

$$\int d\mathbf{x}_k\, g_{j\to k}(\mathbf{x}_{j-1}|\mathbf{x}_k) = 1.0 = \int d\mathbf{x}_{j-1}\, g_{j\to k}(\mathbf{x}_{j-1}|\mathbf{x}_k) \quad (38)$$

The symbol $g_{j\to k}(\mathbf{x}_{j-1}|\mathbf{x}_k)$ is the transition probability density that a configuration $\mathbf{x}_{j-1}$ converts to $\mathbf{x}_k$ in $k - (j - 1)$ time intervals, each of duration dt, extending from t_{j-1} to t_k. The $d\mathbf{x}_2 \ldots d\mathbf{x}_{N-2}$ integrals in the last line of eq 35 can be performed using eq 36 and extensions thereof to convert the extended product of $g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)$ functions to a single function,

$g_{2\to N-1}(\mathbf{x}_1|\mathbf{x}_{N-1})$. Performing the $d\mathbf{x}_{N-1}$ integral of this quantity yields 1.0 according to eq 38. This gives

$$\langle\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\rangle = (Z_0^C Z_1^C Z_N^C)^{-1} Z_0^{in}(z_a)\, e^{-\beta u(z_a,\zeta_0)} \int d\mathbf{x}_1\, e^{-\beta(V(\mathbf{x}_1)+u_1(\mathbf{x}_1))} \int d\mathbf{x}_N\, e^{-\beta(V(\mathbf{x}_N)+u_N(\mathbf{x}_N))}\, \delta(z(\mathbf{x}_N)-z_b) = \frac{Z_0^{in}(z_a)Z_0^{in}(z_b)}{Z_0^C Z_N^C}\, e^{-\beta(u(z_a,\zeta_0)+u(z_b,\zeta_N))} \quad (39)$$

Inserting eqs 34 and 39 into 33 yields, after some cancellation

$$\langle e^{-\beta w_T(t)}\rangle_{z_a\to z_b} = \frac{Z_N^C}{Z_1^C} e^{\beta(u(z_b,\zeta_N)-u(z_a,\zeta_1))} = e^{-\beta(A_t^C-A_0^C-(u(z_b,\zeta(t))-u(z_a,\zeta(0))))} \quad (40)$$

In proceeding from the first to the second line of eq 40, it has been assumed that $\zeta_1 = \zeta_0$, as discussed above. As a consequence of this assumption, $u_1(z(\mathbf{x})) = u(z(\mathbf{x}),\zeta_1) \cong u(z(\mathbf{x}),\zeta_0) = u_0(z(\mathbf{x}))$ and $Z_1^C \cong Z_0^C$. Again, Z_j^C is given by eq 22 and (with $Z_N^C = Z_t^C$) also $A_t^C - A_0^C = -\beta^{-1}\ln(Z_t^C/Z_0^C)$. It is notable that the free energies on the right-hand side of eq 40 pertain not to bare systems with fixed extension, as conjectured by Liphardt et al., but instead to composite systems that exhibit equilibrium fluctuations in extension of the bare system. This circumstance arises from the fact that the initial and final steps are reversible equilibrium steps. The difference between the right-hand side of eq 40 and the conjectured result of Liphardt et al. will be discussed in detail below.

It is useful at this point to derive several more equalities pertaining to this same experiment. The first objective is to evaluate

$$\langle e^{-\beta w_A(t)}\rangle_{z_a\to z_b} = \frac{\langle e^{-\beta w_A(t)}\,\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\rangle}{\langle\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\rangle} \quad (41)$$

The numerator in eq 41 is evaluated by taking the sum in the exponent of the fifth line of eq 13 as the discretized form of $w_A(t)$. Then

$$\langle e^{-\beta w_A(t)}\,\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\rangle = (Z_0^C)^{-1}\int\!\cdots\!\int d\mathbf{x}_N\cdots d\mathbf{x}_0\, e^{-\beta(V(\mathbf{x}_0)+u_0(\mathbf{x}_0))}\, \delta(z(\mathbf{x}_0)-z_a)\, e^{-\beta(u_1(\mathbf{x}_0)-u_0(\mathbf{x}_0))} \frac{e^{-\beta(V(\mathbf{x}_1)+u_1(\mathbf{x}_1))}}{Z_1{}^C}\{\prod_{j=2}^{N-1} e^{-\beta(u_j(\mathbf{x}_{j-1})-u_{j-1}(\mathbf{x}_{j-1}))} g_j(\mathbf{x}_{j-1}|\mathbf{x}_j)\} \frac{e^{-\beta(u_N(\mathbf{x}_{N-1})-u_{N-1}(\mathbf{x}_{N-1}))} e^{-\beta(V(\mathbf{x}_N)+u_N(\mathbf{x}_N))}}{Z_N^C}\,\delta(z(\mathbf{x}_N)-z_b) = (Z_0^C Z_1^C Z_N^C)^{-1} Z_0^{in}(z_a) e^{-\beta u_1(z_a)} \int\int d\mathbf{x}_N\, d\mathbf{x}_{N-1}\, e^{-\beta(V(\mathbf{x}_{N-1})+u_N(\mathbf{x}_{N-1}))}\, e^{-\beta(V_N(\mathbf{x}_N)+u_N(\mathbf{x}_N))}\,\delta(z(\mathbf{x}_N)-z_b) = \frac{Z_0^{in}(z_a)Z_0^{in}(z_b)Z_N^C\, e^{-\beta(u(z_a,\zeta_1)+u(z_b,\zeta_N))}}{Z_0^C Z_1^C Z_N^C} \quad (42)$$

Equation 21 was employed for the reversible first and last steps of the stretching process, and again the Z_j^C are given by eq 22. The third line in eq 42 is obtained from the second by repeated iteration of the balanced mapping condition (eq 12). The Z_N^C factors can be canceled out of eq 42. Likewise, it is assumed that $\zeta_1 = \zeta_0$, as discussed above, so $Z_1^C = Z_0^C$. After implementing these changes, eq 42 is combined with eq 39 in eq 41 to give

$$\langle e^{-\beta w_A(t)}\rangle_{z_a\to z_b} = Z_N^C/Z_0^C = e^{-\beta(A_t^C-A_0^C)} \quad (43)$$

Equation 43 could also have been obtained directly from eq 40 by noting that $u(z_b,\zeta(t))$ and $u(z_a,\zeta(0))$ are constants unaffected by the averaging. Multiplication of both sides of eq 40 by $\exp(+\beta(u(z_b,\zeta(t)) - u(z_a,\zeta(0))))$ then gives $\exp(-\beta(A_t^C - A_0^C))$ on the right, and $\langle\exp(-\beta(w_A(t)))\rangle_{z_a\to z_b}$ on the left, since $w_A(t) = w_T(t) + u(z_b,\zeta(t)) - u(z_a,\zeta(0))$. The utility of the densities in eqs 34 and 42 will be discussed subsequently.

The average value of $\langle w_T^{rev}\rangle$ for the same conditions is evaluated as follows

$$\langle w_T^{rev}\rangle_{z_a\to z_b} = \frac{\langle\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\{(-)\sum_{k=1}^{N}(u_k(\mathbf{x}_k)-u_k(\mathbf{x}_{k-1}))\}\rangle}{\langle\delta(z(\mathbf{x}_0)-z_a)\delta(z(\mathbf{x}_N)-z_b)\rangle} \quad (44)$$

The denominator in eq 44 has already been evaluated in eq 39. The numerator in eq 44 is evaluated by the same general protocol employed in eq 23. The presence of the delta functions has two consequences: (1) every term on the right-hand side of eq 23 is multiplied by the factor, $(Z_0^{in}(z_a)Z_0^{in}(z_b)/(Z_0^C Z_N^C))\exp[-\beta(u(z_a,\zeta_0) + u(z_b,\zeta_N))]$ and (2) the quantity $\langle u_1(\mathbf{x}_0)\rangle$ is replaced by $u_1(z_a) = u(z_a,\zeta_0)$ and $\langle u_N(\mathbf{x}_N)\rangle$ is replaced by $u_N(z_b) = u(z_b,\zeta_N)$. The approximation $\zeta_1 = \zeta_0$ was employed again. *With these modifications*, the result in eq 23 is combined with that in (44) to give

$$\langle w_T^{rev}\rangle_{z_a\to z_b} = u(z_a,\zeta_0) + \sum_{k=1}^{N-1}\langle u_{k+1}(\mathbf{x}_k)\rangle_k - \langle u_k(\mathbf{x}_k)\rangle_k - u(z_b,\zeta_N) = \langle w_A^{rev}\rangle_{z_a\to z_b} - (u(z_b,\zeta_N) - u_A(z_a,\zeta_0)) \quad (45)$$

where in this case

$$\langle w_A^{rev}\rangle_{z_a\to z_b} = \sum_{k=1}^{N-1}\langle u_{k+1}(\mathbf{x}_k)\rangle_k - \langle u_k(\mathbf{x}_k)\rangle_k \quad (46)$$

and $\langle u_l(\mathbf{x}_k)\rangle_k$ is defined in eq 24. The sum in $\langle w_A^{rev}\rangle$ generally begins at $k = 0$, but in this case it is assumed that $u_1(\mathbf{x}_0) = u(z_a,\zeta_1) = u(z_a,\zeta_0) = u_0(\mathbf{x}_0)$, so the $k = 0$ term vanishes. Comparison of eqs 46 and 26 gives

$$\langle w_A^{rev}\rangle_{z_a\to z_b} = A_N^C - A_0^C = \langle w_A^{rev}\rangle \quad (47)$$

where $\langle w_A^{rev}\rangle$ pertains to an equilibrium process in which ζ is varied *slowly* from ζ_0 to ζ_N (or ζ_t), but the averaging is performed over all initial and final molecular extensions, which are *not* constrained to z_a and z_b, respectively. Substituting the first equality in eq 47 (with $A_N^C = A_t^C$) into eq 45 gives

$$\langle w_T^{rev}\rangle_{z_a\to z_b} = A_t^C - A_0^C - (u(z_b,\zeta(t)) - u(z_a,\zeta(0))) \quad (48)$$

Comparison of eqs 40 and 48 then yields

$$-\beta^{-1}\ln(\langle\exp(-\beta w_T(t))_{z_a\to z_b}\rangle) = \langle w_T^{rev}\rangle_{z_a\to z_b} \quad (49)$$

Comparison of eqs 43 and 47 yields

$$-\beta^{-1}\ln(\langle\exp(-\beta w_A(t))_{z_a\to z_b}\rangle) = \langle w_A^{rev}\rangle_{z_a\to z_b} \quad (50)$$

Thus, under the conditions of this experiment, the trajectory averages of these exponential functions of the nonequilibrium works, $w_T(t)$ and $w_A(t)$, are similarly related to the respective equilibrium values of those same works.

Comparison of Predictions with the Experiments of Liphardt et al. The elegant experiments of Liphardt et al. demonstrated two important results: (1) $\langle\exp(-\beta w_T(t))\rangle_{z_a\to z_b}$ is independent of the rate of elongation, $\partial\zeta(t)/\partial t$, of the distance between the pipet and the center of the trap, well into the regime where the average work, $\langle w_T(t)\rangle$, depends strongly on $\partial\zeta(t)/\partial t$. Equation 40 is completely consistent with that observation. (2) For reversible slow stretching experiments, $-\beta^{-1}\ln[\langle\exp(-\beta w_T(t)]\rangle_{z_a\to z_b})$ is very nearly equal to $\langle w_T^{rev}\rangle_{z_a\to z_b}$. Equation 49 is consistent with that observation. Thus, these new equalities for the work, $w_T(t) = \int_{z_a}^{z_b} F_z(t)\,dz(t)$, that is transferred from the external potential to the bare system are in agreement with the experimental observations.

According to the conjecture of Liphardt et al.

$$\langle\exp(-\beta w_T(t))\rangle_{z_a\to z_b} = \exp(-\beta(A_0^{in}(z_b) - A_0^{in}(z_a))) = Z_0^{in}(z_b)/Z_0^{in}(z_a) \quad (51)$$

This differs from eq 40 in two significant respects: (1) The free energies appearing on the right-hand side of eq 40 apply to the composite system at pipet-to-trap-center distances, $\zeta(t)$ and $\zeta(0)$, whereas the right-hand side of eq 51 pertains to the bare system at the initial and final molecular extensions, z_b and z_a. In particular, the free energies A_t^C and A_0^C in eq 40 contain the external potential and also the effects of fluctuations in the molecular extension, whereas the free energies in eq 51 contain only the bare potential and admit no fluctuations in molecular extension. (2) The difference in external potential energy between the z_b and z_a extensions is subtracted from $A_t^C - A_0^C$. The difference between the right-hand sides of eqs 40 and 51 is illustrated by the following explicit example.

A Comparison between Equations 40 and 51. The first objective is to relate Z_N^C/Z_0^C to $Z_0^{in}(z_b)/Z_0^{in}(z_a)$ for a hypothetical circumstance. The composite partition functions are rewritten as

$$Z_j^C = \int dz \int d\mathbf{x}_j\, e^{-\beta(V(\mathbf{x}_j)+u(z(\mathbf{x}_j),\zeta_j))}\delta(z(\mathbf{x}_j) - z) = \int dz\, Z_0^{in}(z) e^{-\beta u(z,\zeta_j)} \quad (52)$$

where $Z_0^{in}(z)$ is given by eq 7. $Z_0^{in}(z)$ is rewritten as $\exp(-\beta(-)\beta^{-1}\ln(Z_0^{in}(z)))$, and Z_j^C is rewritten as

$$Z_j^C = \int dz\, e^{-\beta(-\beta^{-1}\ln(Z_0^{in}(z))+u(z,\zeta_j))} \quad (53)$$

The optical trap potential is assumed to be harmonic

$$u(z,\zeta_j) = (g/2)(z - \zeta_j)^2 \quad (54)$$

This is expanded to second order about the initial position z_a, when $j = 0$, to obtain

$$u(z,\zeta_0) = (g/2)(z_a - \zeta_0)^2 + g(z_a - \zeta_0)(z - z_a) + (g/2)(z - z_a)^2 \quad (55a)$$

A similar expansion around z_b, when $j = N$, yields

$$u(z,\zeta_N) = (g/2)(z_b - \zeta_N)^2 + g(z_b - \zeta_N)(z - z_b) + (g/2)(z - z_b)^2 \quad (55b)$$

In fact, these two power series have no higher terms. The quantity $-\beta^{-1}\ln(Z_0^{in}(z))$ is similarly expanded about z_a to second order to obtain

$$-\beta^{-1}\ln(Z_0^{in}(z)) = a_0 + b_0(z - z_a) + (c_0/2)(z - z_a)^2 \quad (56a)$$

and again about z_b to obtain

$$-\beta^{-1}\ln(Z_0^{in}(z)) = a_N + b_N(z - z_b) + (c_N/2)(z - z_b)^2 \quad (56b)$$

where

$$a_0 = -\beta^{-1}\ln(Z_0^{in}(z_a)) \quad (57a)$$

$$a_N = -\beta^{-1}\ln(Z_0^{in}(z_b)) \quad (57b)$$

and b_0 and b_N are the first derivatives, and c_0 and c_N are the second derivatives of $-\beta^{-1}\ln(Z_0^{in}(z))$ at, respectively, z_a and z_b. Incorporating eqs 55a, 56a, and 57a into 53 for $j = 0$ gives

$$Z_0^C = Z_0^{in}(z_a)e^{-\beta(g/2)(z_a-\zeta_0)^2} \int dz\, e^{-\beta((b_0+g(z_a-\zeta_0))(z-z_a)+((g+c_0)/2)(z-z_a)^2)} \quad (58)$$

The value of z for which the total effective potential energy in the exponent of eq 58 is minimal is found by operating on that potential with $\partial/\partial z$ and setting the result to 0

$$b_0 + g(z_a - \zeta_0) + (g + c_0)(z - z_a) = 0 \quad (59)$$

For such a quadratic potential, the average value of z will be the most probable value, which occurs at the minimum of the effective potential in eq 58. However, the average, or most probable, value of z, when $\zeta = \zeta_0$, has already been assumed to be z_a. When $z = z_a$, the last term in eq 59 vanishes and one finds $b_0 + g(z_a - \zeta_0) = 0$. This relation gives the value of z_a in terms of the relevant potential parameters and also causes the coefficient of the $(z - z_a)$ term in the exponent of eq 58 to vanish. The integral in eq 58 is now readily completed to yield

$$Z_0^C = Z_0^{in}(z_a)e^{-\beta(g/2)(z_a-\zeta_0)^2}\left(\frac{2\pi}{\beta(c_0+g)}\right)^{1/2} \quad (60a)$$

A similar analysis gives

$$Z_N^C = Z_0^{in}(z_b)e^{-\beta(g/2)(z_b-\zeta_N)^2}\left(\frac{2\pi}{\beta(c_N+g)}\right)^{1/2} \quad (60b)$$

Combining eqs 60a and 60b gives

$$\frac{Z_N^C}{Z_0^C} = \frac{Z_0^{in}(z_b)}{Z_0^{in}(z_a)} \exp\left(-\beta\left(\left(\frac{g}{2}(z_b - \zeta_N)^2 - \frac{g}{2}(z_a - \zeta_0)^2\right)\right)\left(\frac{c_0 + g}{c_N + g}\right)^{1/2} \quad (61)$$

Inserting this result into eq 40 yields

$$\langle e^{-\beta w_T(t)}\rangle_{z_a \to z_b} = \frac{Z_0^{in}(z_b)}{Z_0^{in}(z_a)}\left(\frac{c_0 + g}{c_N + g}\right)^{1/2} \quad (62)$$

For this simple case, when the power series expansions of $-\beta^{-1}\ln(Z_0^{in}(z))$ about z_a and z_b do not extend beyond the second-order terms and the trap potential is harmonic, the right-hand side of eq 62 differs from that of eq 51 (conjectured by Liphardt et al.) by the correction factor $((c_0 + g)/(c_N + g))^{1/2}$. This extra factor is the ratio of partition functions for two effective springs, one with force constant $c_N + g$ and the other with force constant $c_0 + g$. Only in the event that the curvature of $-\beta^{-1}\ln(Z_0^{in}(z))$ is the same at z_b as at z_a will $c_N = c_0$, in which case this factor takes the value 1.0. However, stretching experiments are typically performed in a domain outside the harmonic region of the effective potential (or potential of mean force), $-\beta^{-1}\ln(Z_0^{in}(z))$, of the bare system, so $c_0 \neq c_N$ in such cases. Of course, if the external optical trap potential is very much stiffer than the bare molecule, then $g \gg c_0$, c_N, and the factor $((c_0 + g)/(c_N + g))^{1/2} \cong 1.0$. However, in the experiments of Liphardt et al., the molecule is actually considerably (~6-fold) stiffer than the optical trap.

Estimated Errors Arisng from Equation 51. From fitting their data in the reversible regions, Liphardt (personal communication) estimates that $g = 0.1$ pN/nm, $c_0 = 0.53$ pN/nm, and $c_N = 0.65$ pN/nm. In this case $((c_0 + g)/(c_N + g))^{1/2} = 0.92$. This is a modest correction to the partition function ratio in eq 48. Accordingly, the value of $A_t(z_b) - A_0(z_a)$ obtained from eq 51 should be augmented by the correction term $((\beta^{-1}/2)\ln((c_0 + g)/(c_N + g)) \cong -(0.08)$ kT, which is 0.14% of the left-hand side of eq 45, namely, $-\beta^{-1}\ln\langle\exp(-\beta w_T(t))\rangle_{z_a \to z_b} = 60$ kT. These conclusions are based on a truncation of the expansion of the effective molecular potential at second order, which might not be entirely correct.

More generally, when $(c_0 + g)/(c_N + g) \leq 10$ and $-\beta^{-1}\ln(\langle\exp(-\beta w_T)\rangle) \geq (10.0)$ kT, then eq 51 will overestimate $A_0^{in}(z_b) - A_0^{in}(z_a)$ by less than 11%. Thus, the relation (eq 51) conjectured by Liphardt et al. is expected to provide a reasonably good approximation for $A_0^{in}(z_b) - A_0^{in}(z_a)$ not only in their study but in other circumstances as well. Nevertheless, the partition function ratio, which directly reflects the relative probability of finding the bare system at z_a or z_b, may exhibit far greater errors, as much as 10-fold within the range specified above. Thus, the approximate relation of Liphardt et al. in eq 51 is expected to be useful primarily for estimating free-energy differences and the corresponding reversible works but may introduce significant, or even very large, errors into the partition function ratios.

Connection of Equation 34 to Other Equalities. Attention is now turned to the two-dimensional density in eq 34. After multiplying both sides by $\exp(+\beta(u(z_a,\zeta_0)))$, which can be incorporated inside the averaging integral as $\exp(+\beta(u(z(\mathbf{x}_0),\zeta_0)))$, setting $\zeta_1 = \zeta_0$, and integrating over z_a by using eq 52 on the right-hand side, one obtains

$$\langle\exp(-\beta(w_T(t) - u(z(\mathbf{x}_0),\zeta_0)))\,\delta(z(\mathbf{x}_N) - z_b)\rangle = Z_0^{in}(z_b)/Z_0^C = \exp(-\beta A_0^{in}(z_b)) \quad (63)$$

Equation 63 is just a rewritten form of eq 6. This is expected because the exponential factor has been modified to be the same as in eq 6 and the integral over dz_a completes the averaging over all canonically weighted initial conditions. Equation 63 is valid under the more general conditions pertaining to eq 6. In particular, the trajectories are not required to be reversible anywhere, except for $t \leq 0$.

An alternative expression can also be derived from eq 34. After multiplying both sides by $\exp(-\beta(u(z_b,\zeta_0) - u(z_a,\zeta_0)))$, which can be incorporated inside the averaging integral as $\exp(-\beta(u(z(\mathbf{x}_N),\zeta_0) - u(z(\mathbf{x}_0),\zeta_0)))$, setting $\zeta_1 = \zeta_0$, and integrating over both z_a and z_b, using eq 52 in each case, one obtains

$$\langle\exp(-\beta(w_T(t) + u(z(\mathbf{x}_N),\zeta_0) - u(z(\mathbf{x}_0),\zeta_0)))\rangle = 1.0 \quad (64)$$

Equation 64 is just a rewritten form of eq 17. This is expected because the exponential factor has been modified to be the same, the integral over dz_a completes the averaging over all canonically weighted initial conditions and the integral over dz_b completes the averaging over all possible trajectory termini. Equation 64 is valid under the more general conditions pertaining to eq 18. In particular, the trajectories are not required to be reversible anywhere, except for $t \leq 0$.

A Protocol for Determining the Free-Energy Profile in a Stretching Experiment. The desired quantity is the difference in profile free energies, $A_0^{in}(z_b) - A_0^{in}(z_a) = -\beta^{-1}\ln(Z_0^{in}(z_b)/Z_0^{in}(z_a))$, where $z_b > z_a$. Consider a fixed rate of extension, $d\zeta(t)/dt$ of the pipet−trap-center distance, beginning always from the same initial $\zeta_0 < z_a$ at $t = 0$. The trajectory is regarded as reversible at the given pulling speed in the vicinity of ζ_0 but not necessarily at any other (pipet to trap center) distance. Trajectories of $\zeta(t)$ are initiated from ζ_0 at random, without regard for the initial molecular extension, $z(\mathbf{x}_0) = z(\mathbf{x}(0))$, which is *not* z_a in this discussion. Work transfer from the external potential to the bare molecular system is calculated for all observed trajectories over the same *time range*, 0 to t, by

$$w_T(t) = \int_{z(\mathbf{x}_0)}^{z(\mathbf{x}_t)} F(z(\mathbf{x}_{t'}),\zeta_{t'})\,dz(\mathbf{x}_{t'}) \quad (65)$$

where $z(\mathbf{x}_0)$ and $z(\mathbf{x}_t) = z(\mathbf{x}(t))$ are the variable extensions at $t = 0$ and t, $dz(\mathbf{x}_t') = z(\mathbf{x}_j) - z(\mathbf{x}_{j-1})$ is the change in extension during the jth time interval, and $F(z(\mathbf{x}_t'),\zeta t') = F(z(\mathbf{x}_j),\zeta_{j-1})$ is the force prevailing throughout the jth time interval, which extends from t_{j-1} to t_j. The average value of the molecular extension, $\langle z(\mathbf{x}_j)\rangle$, for the set of collected trajectories can be calculated and plotted as a function of trajectory time, t_j. Then the time t_i required for the average of the molecular extensions $\langle z(\mathbf{x}(t_i))\rangle$ to reach a selected value z_i can be estimated by interpolation, and $w_T(t_i)$ can be calculated for each trajectory from 0 to t_i from its temporal record of forces and molecular displacements. Also, for each trajectory the quantities, $u_0(\mathbf{x}_0) = u(z(\mathbf{x}_0),\zeta(0))$ and $u_0(\mathbf{x}_i) = u(z(\mathbf{x}_{t_i}),\zeta(0))$, can be calculated. If the entire experiment is conducted within the linear range of the optical trap, with potential given by eq 54, then $u_0(\mathbf{x}_0) = (g/2)(z(\mathbf{x}_0) - \zeta(0))^2$ and $u_0(\mathbf{x}_i) = (g/2)(z(\mathbf{x}(t_i)) - \zeta(0))^2$. For a calibrated trap potential, g is known and $z(\mathbf{x}_0) - \zeta(0)$ is obtained directly from the measured force (or deflection of the trapping beam) at $t = 0$. However, the system extension, $z(\mathbf{x}(t_i))$, at time t_i is normally so large that it, or values anywhere near it, are not normally observed (as an extreme fluctuation) at $t = 0$, when

$\zeta = \zeta(0)$. Hence $u_0(\mathbf{x}_i) = u_0(\mathbf{x}_{t_i},\zeta(0))$ will in most cases have to be dead reckoned from independent measurements of $\zeta(0)$ and $z(\mathbf{x}_{t_i})$, or perhaps more accurately from the measured displacement at time t_i, namely, $z(\mathbf{x}_{t_i}) - \zeta(t_i)$ and $\zeta(t_i) - \zeta(0)$ for that particular trajectory. Next a suitable range, $\Delta z_i = \Delta z(\mathbf{x}_{t_i})$, of molecular extensions at time t_i is defined so as to be centered at z_i and to include some or all of the region with the highest density of (trajectory) extensions $z(\mathbf{x}_{t_i})$. If one has very many trajectories, it may be feasible to mark the $z(\mathbf{x}_{t_i})$ axis off into several adjacent ranges of the same size. The most suitable range size is likely to depend on the experimental circumstances. The molecular trajectories that terminate in each range are identified, and the associated weight, $\exp(-\beta(w_T(t_i) - u(z(\mathbf{x}_0),\zeta(0))))$, is calculated for each trajectory in that range. These weights are summed for all trajectories in that range, which may comprise only a fraction of the total trajectories. The resulting sum is then divided by the *total* number of trajectories η terminating in *all* ranges at t_i, and by the range size, Δz_i, to obtain the density on the left-hand side of eq 63. That is, the experimental estimate of the left-hand side of eq 63 is given by

$$\langle e^{-\beta(w_T(t_i)-u(z(\mathbf{x}_0),\zeta(0)))}\,\delta(z(\mathbf{x}_{t_i} - z_i))\rangle = \left(\frac{1}{(\eta\Delta z_i)}\right)\sum_{\alpha=1}^{\nu} e^{-\beta(w_{T\alpha}(t_i)-(g/2)(z_{0\alpha}-\zeta(0))^2)} \quad (66)$$

where α denotes a particular trajectory with its $z(\mathbf{x}_i)$ in a particular range Δz_i at time t_i, $z_{0\alpha} = z(\mathbf{x}_0)_\alpha$ denotes the initial molecular extension of the αth trajectory at $t = 0$, and ν is the number of trajectories with $z(\mathbf{x}_i)_\alpha$ in the particular range Δz_i at time t_i. The density in eq 66 should be independent of range size, Δz_i, so the most populous range should contain sufficient trajectories that halving the range size reduces the number of trajectories by about half, if possible. For the most populous range, the preceding protocol provides an estimate of $Z_0^{in}(z_i)/Z_0^C$ on the right-hand side of eq 66. Should sufficient trajectories be available to examine other ranges centered at $z_i + n\Delta z_i$, where $n = \pm1, \pm2, ...$, then the same protocol applied to those ranges may provide statistically significant estimates of $Z_0^{in}(z_i + n\Delta z_i)/Z_0^C$. The same protocol is repeated for a later time t_f at which $\langle z(\mathbf{x}_{t_f})\rangle = z_f$. For the most populous range, this protocol provides an estimate of $Z_0^{in}(z_f)/Z_0^C$. For adjacent ranges, repeating this same protocol may provide statistically significant estimates of $Z_0^{in}(z_f + n\Delta z_f)/Z_0^C$, $n = \pm1, \pm2, ...$, provided that there are sufficient trajectories. Especially when the number ν of trajectories in the most populous range is small, it is important to use the same range size $\Delta z_f = \Delta z_i$ at all times examined, so all densities are on the same footing. With very few trajectories, one is likely to have statistically significant estimates for only a single range at each time, t_i and t_f. In that case, the common range size $\Delta z_f = \Delta z_i$ should be chosen so as to include the same number of trajectories in both Δz_i centered near z_i and Δz_f centered near z_f. The estimate for $Z_0^{in}(z_f)/Z_0^C$ can be divided by that for $Z_0^{in}(z_i)/Z_0^C$ to provide an estimate for $Z_0^{in}(z_f)/Z_0^{in}(z_i)$, and ultimately for $A_0^{in}(z_f) - A_0^{in}(z_i) = -\beta^{-1}\ln(Z_0^{in}(z_f)/Z_0^{in}(z_i))$.

This protocol is similar to, but more explicit than, that suggested by Hummer and Szabo for the case of position integration, which requires evaluation of $w_T(t) - u(z(\mathbf{x}_0),\zeta(0)) = \int F_z(t)dz_t - u(z(\mathbf{x}_0),\zeta(0))$.[36] The case of time integration requires evaluation of $w_A(t) - u(z(\mathbf{x}_t),\zeta(t))$. The position integration protocol appears to be potentially more accurate because there is normally less error in $\zeta(0)$ than in $\zeta(t)$ due to the slow or vanishing pulling rate required in the reversible region at $t \le 0$. Moreover, because $\zeta(t)$ generally exceeds $\zeta(0)$ in a stretching experiment, the value of the external potential energy is larger at time t and therefore is more sensitive to errors in the null-force position $\zeta(t)$.

It is also desired to test the adequacy of the sampling provided by the observed trajectories. The suggested protocol is related to that just described but differs in two main regards: (1) the weight of each trajectory is now given by $\exp(-\beta(w_T(t_i) + u(z(\mathbf{x}_{t_i}),\zeta(0)) - u(z(\mathbf{x}_0),\zeta(0))))$ and (2) the average is simply taken over all trajectories, regardless of where they terminate, and there is no need to define the ranges themselves, or to calculate a density by dividing by a range. For the time t_i, the new associated weights are calculated for each trajectory. These weights are simply summed for all trajectories, and the resulting sum is divided by the total number of trajectories, η, to obtain the left-hand side of eq 64. Hence, the experimental estimate of the left-hand side of eq 64 is given by

$$\langle e^{-\beta(w_T(t_i)+u(z(\mathbf{x}_{ti}),\zeta(0))-u(z(\mathbf{x}_0),\zeta(0)))}\rangle = \left(\frac{1}{\eta}\right)\sum_{\alpha=1}^{\eta} e^{-\beta(w_{T\alpha}(t_i)+(g/2)(z(\mathbf{x}_{ti})_\alpha-\zeta(0))^2-(g/2)(z(\mathbf{x}_0)_\alpha-\zeta(0))^2)} \quad (67)$$

where α denotes a particular trajectory, $z(\mathbf{x}_0)_\alpha$ denotes the initial molecular extension of the αth trajectory at $t = 0$, and $z(\mathbf{x}_{t_i})_\alpha$ denotes the molecular extension of the αth trajectory at time t_i. According to eq 64, the right-hand side of eq 67 should equal 1.0. If it deviates significantly from 1.0, that would indicate that the trajectory sampling is insufficient to achieve this required equality and *might* imply that the trajectory sampling is also inadequate to provide a reliable estimate of $Z_0^{in}(z_f)/Z_0^{in}(z_i)$. The sampling requirements of the two equalities (eqs 63 and 64) are not identical because the weights of the trajectories are very different in the two cases. The proposal that eq 64, evaluated via eq 67, might provide a useful test of sufficient sampling also in the case of eq 63, evaluated via eq 66, is a plausible, but so far untested, conjecture. Numerical studies to test this conjecture are currently in progress.

Connection to Macroscopic Thermodynamics. In thermodynamics, it is common to consider the variation in properties of a bare system when an extensional parameter, such as its length, area, or volume is varied. To physically hold the system at a precise value of the extensional parameter, which admits no significant fluctuations, the external potential function must arise from a spring with a sufficiently large force constant to suppress fluctuations in that extensional parameter. In other words, a sufficiently stiff external spring will render fluctuations in the extensional parameter so costly in an energetic sense that the probability of a fluctuation of significant amplitude is drastically reduced. A sufficiently stiff spring is one whose force constant, g, far exceeds that of the bare system at the current average value of its extension. Specifically, g far exceeds c_0 and c_N in eqs 56a and 56b, respectively. In this case, the Taylor series for the effective potential of the bare system in the vicinity of z_a and z_b, respectively, can be safely truncated at the second-order terms, because the external potential prevents the displacements $z - z_a$ and $z - z_b$ from reaching such a size that the second and higher order terms are significant. The analysis leading from eqs 56a and 56b to 62 thus becomes exact in the limit of large g. Moreover, in this same large g limit, the factor $((c_0 + g)/(c_N + g))^{1/2} = 1.0$ and

$$\langle e^{-\beta w_T(t)}\rangle_{z_a\to z_b} = Z_0^{in}(z_b)/Z_0^{in}(z_a) = e^{-\beta(A_N{}^{in}(z_b)-A_0{}^{in}(z_a))} \quad (68)$$

When fluctuations in the bare system are suppressed by such a

strong external spring, the system moves always along the same smooth trajectory from z_a to z_b, but the force applied by the spring may fluctuate considerably during that process. It is still implicit in eq 68 that the initial and final steps of the path are equilibrated steps. Thus, the bare system begins in one equilibrium state with extension z_a and ends in another equilibrium state with extension z_b but may occupy nonequilibrium states in between. In such a case, eq 68 is the appropriate relation connecting the nonequilibrium transferred work, $w_T(t) = \int_{z_a}^{z_b} F(z_t)\, dz_t$, to the change in Helmholtz free energy of the bare system. Again invoking the inequality $\langle \exp(y) \rangle \geq \exp(\langle y \rangle)$, one finds that $\langle w_T \rangle_{z_a \to z_b} \geq A_0^{in}(z_b) - A_0^{in}(z_a)$, which is a standard relation of macroscopic thermodynamics. In this case, the equality holds for a reversibly slow process, and $\langle w_T^{rev} \rangle_{z_a \to z_b}$ may be either positive or negative, depending upon the direction of the process (or equivalently on the sign of $A_0^{in}(z_b) - A_0^{in}(z_a)$).

An essential point of this discussion is that an appropriately strong external spring is required to precisely constrain the values of the extensional parameters such as length, area, and volume. The relevant condition is that the curvature of the spring potential must far exceed that of the effective system potential. In this case, which typically prevails for macroscopic systems whose extensional parameters are well defined, the relation conjectured by Liphardt et al., namely, eq 68 above, becomes valid.

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