Experimental free-energy measurements of kinetic molecular states using fluctuation theorems

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Recent advances in non-equilibrium statistical mechanics and single-molecule technologies have made it possible to use irreversible work measurements to extract free-energy differences associated with the mechanical (un)folding of molecules. To date, free-energy recovery has been focused on native (or equilibrium) molecular states, but free-energy measurements of kinetic states have remained unexplored. Kinetic states are metastable, finite-lifetime states that are generated dynamically, and play important roles in diverse physical processes. In biophysics, there are many examples in which these states determine the fate of molecular reactions, including protein binding, enzymatic reactions, as well as the formation of transient intermediate states during molecular-folding processes. Here we demonstrate that it is possible to obtain free energies of kinetic states by applying extended fluctuation relations, using optical tweezers to mechanically unfold and refold deoxyribonucleic acid (DNA) structures exhibiting intermediate and misfolded kinetic states.

Kinetic states are observed under non-equilibrium conditions and have higher free energies than native states. Yet, they can be crucial, as shown by the role that misfolded proteins play in numerous severe diseases¹. The measurement of the free energy of formation of kinetic states is therefore a central question in biophysics. Recent theoretical developments known as fluctuation relations²–⁶ have been applied to extract free-energy differences of equilibrium states from irreversible work measurements. Applications include the measurement of the free energy of formation of ribonucleic acid (RNA) hairpins², the determination of the stability of native domains in proteins³, the measurement of mechanical torque in rotary motors⁴, the conversion of information into work in systems under feedback control⁵; and the recovery of free-energy landscapes from unidirectional work measurements⁶,¹¹,¹².

The characterization of kinetic states under non-equilibrium conditions remains a challenging problem. Here we use a recently introduced extended fluctuation relation (EFR) to extract free energies of kinetic states and thermodynamic branches using irreversible work measurements³,¹⁴. In the EFR, a kinetic state is a partially equilibrated region of configurational space, meaning that during a finite timescale the system is confined and thermalized within that region¹⁵. This is mathematically described by a Boltzmann–Gibbs distribution restricted to configurations contained in that region (Fig. 1a).

Let A, B denote any two kinetic states and λ, a control parameter. We consider a forward (F) non-equilibrium process, where the system starts in partial equilibrium in A at λ₀, and its time-reversed process (R), where the partial equilibrium condition is required over B at λ₁. In the F process λ varies from λ₀ to λ₁ during a time t according to a predetermined protocol λ(t). For the R process the time-reversed protocol λ(τ − t) is used. The EFR reads¹⁴:

\[
\frac{\phi^{A\rightarrow B}_F}{\phi^{A\rightarrow B}_R} \frac{P^{A\rightarrow B}(W)}{P^{A\rightarrow B}(-W)} = \exp \left( \frac{W - \Delta G_{AB}}{k_B T} \right)
\]

where W is the work performed along a process; ΔG_{AB} = G_B(λ₁) − G_A(λ₀) is the free-energy difference between kinetic states B at λ₁ and A at λ₀; P^{A\rightarrow B}(W) (P^{A\rightarrow B}(-W)) denotes the partial work distribution for the F (R) process over the fraction of paths ϕ^{A\rightarrow B} (ϕ^{A\rightarrow B}) starting in A (B) at λ₀ (λ₁) and ending in B (A) at λ₁ (λ₀); k_B is the Boltzmann constant and T the temperature of the environment.

We applied equation (1) to extract free-energy differences of kinetic states from mechanical unfolding/folding experiments performed on DNA hairpins, which are model systems easy to design and synthesize. Their free energies of formation can be predicted using the nearest-neighbour (NN) model with the unified-oligonucleotide (UO) set of parameters¹⁶,¹⁷ or with recently derived energies from unzipping experiments¹⁸ (Methods and Supplementary Section S1). Molecules exhibiting two types of kinetic state were investigated (Fig. 1b): molecules I₁ and I₂ have intermediate kinetic states on-pathway to the native state, and molecules M₁ and M₂ have misfolded kinetic states off-pathway to the native state. To establish the validity of our approach, we first show results for molecules I₁ and M₁, where free energies measured from the EFR applied to non-equilibrium pulling experiments can be compared with free energies obtained from equilibrium hopping experiments. The method is then applied to molecules I₂ and M₂, where irreversibility or low signal-to-noise ratio in hopping traces preclude equilibrium-based free-energy measurements.

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The experimental set-up is shown in Fig. 1c (refs 18,19; Methods). We steer the position of the optical trap up and down to mechanically unfold and refold the DNA hairpin, and measure the force $f$ acting on the hairpin as a function of the relative trap–pipette distance, which is the control parameter $\lambda$ (ref. 20). We measure the work as the area below the force–distance curve (hereafter referred to as the FDC, Fig. 2a inset) along many trajectories. Throughout this paper unfolding (folding) corresponds to the $F$ ($R$) process.

First we apply equation (1) to hairpin I1 characterized by three conformational states (Fig. 1b and Supplementary Section S1): native (N), intermediate (I) and unfolded (U). Experimental hopping traces measured under equilibrium conditions and non-equilibrium FDCs exhibit three force branches corresponding to the three states (Fig. 2a and Supplementary Movie SV11 and Sections S2 and S3). Figure 2b shows the partial work distributions measured from a collection of FDCs by taking $\lambda_0 = 0$, where $\lambda = N, I$ and $\lambda_0 = 55.6$ nm, where the three states are observed (B = N, I or U). These partial work distributions satisfy equation (1) (Supplementary Section S4). Hysteresis effects are stronger for $B = I, U$ than for $B = N$, as the timescale related to the pulling protocol is typically shorter than the timescale for crossing the kinetic barrier separating two states. The acceptance ratio method21,22 applied to extract the free-energy differences between states gives $\Delta G_{NN} \approx \Delta G_{NI}$, and $\Delta G_{NI}$ lies 2 $k_B T$ above (Fig. 2c, Methods). Figure 2d shows the reconstruction of the three thermodynamic branches by fixing $\lambda_0 = 0$ and varying $\lambda_1$ between 45 and 65 nm. The vertical dashed–dotted line at $\lambda_1 = 55.6$ nm indicates the coexistence point of N and U. The full equilibrium free-energy of the system, defined as $\Delta G = -k_B T \log(e^{-\Delta G_{NI}/k_B T} + e^{-\Delta G_{NI}/k_B T} + e^{-\Delta G_{NI}/k_B T})$, has also been measured (Fig. 2d, black line). The right inset in Fig. 2d shows the free energy of each state measured relative to $\Delta G$. For $\lambda < \lambda_c$, $\lambda > \lambda_c$ N(U) is the most stable state, whereas I is never the absolute free-energy minimum for any $\lambda$. The left inset in Fig. 2d shows the contribution of $\phi_{N}^{x-b}/\phi_{N}^{x-b}$ to the measured free energies throughout the $\lambda$ range. Dropping this term or misidentifying states along the FDC leads to wrong free-energy predictions (Supplementary Sections S5 and S6).

By subtracting the elastic contributions due to stretching the handles and the released single-stranded (ss) DNA (Methods and Supplementary Section S7) we extract the free energies of formation of the different structures with respect to the random coil state at zero force. We get $\Delta G_{NN}^{0} = 55 \pm 3 k_B T$ and $\Delta G_{NI}^{0} = 30 \pm 3 k_B T$, in agreement with free-energy predictions and results from equilibrium-based hopping experiments (Table 1).

Next we study hairpin M1, which can fold into two unrelated structures (Fig. 1b and Supplementary Section S1): the native (N) and the misfolded (M). Equilibrium hopping experiments exhibit very fast kinetics and two clearly separated hopping regions: in one region N coexists with an intermediate state on-pathway; in the other region M and U coexist with another intermediate (Supplementary Section S2). For reasons of simplicity, we chose not to characterize these intermediate states. In non-equilibrium experiments, two FDC patterns are identified, corresponding to the two structures (Fig. 3a and Supplementary Movie SVM1 and Section S3). In contrast to the unfolding/folding cycles that start and end in N, those that start and end in M show almost no hysteresis (Fig. 3b), indicating low kinetic barriers between M and U. Owing to kinetic competition of loop formation between M and N, M has a basin of attraction larger than N during folding (≈80% of folding trajectories end in M). In addition, M has lower thermodynamic stability and larger molecular extension at low forces (Fig. 2a inset).

In Fig. 3c we apply the acceptance ratio method to recover the free-energy differences between U at $\lambda_1 = 130$ nm and M (lower set of measurements) or N (upper set) at $\lambda_0 = 0$ nm. By subtracting the handles and ssDNA contributions we extract the free energy of formation of each structure at zero force, obtaining $\Delta G_{MU}^{0} = 47 \pm 2 k_B T$ and $\Delta G_{N}^{0} = 62 \pm 3 k_B T$. The distribution of free energies for different molecules and pulling speeds is shown in Fig. 3d. The difference between the average of both distributions is in agreement with predictions based on the NN model and with results from equilibrium-based hopping experiments (Table 1).
Hairpin I2 has two intermediate states on-pathway, hereafter referred to as I and I' (ref. 23 and Supplementary Section S1). In pulling experiments, four force branches—corresponding to states N, I, I', and U—are distinguished (Fig. 4a and Supplementary Movie SV12 and Sections S3 and S4). To measure forward and reversed partial work distributions for the four states we pull the molecule back and forth between λ = 0 (where the molecule is in equilibrium at N) and λ = 183 nm (where the molecule is partially equilibrated at states N, I, I', and U; Fig. 4a,b). This protocol is subtly different from the standard pulling experiments we did for the rest of molecules (I1, M1, M2), where the molecule is never in an intermediate state at initial and final values of λ. Owing to the larger hysteresis exhibited by this molecule (Supplementary Section S7), the standard protocol does not generate reverse trajectories that sample all four states for any value of λ.

In Table 1 we show the values of the free energy of formation of the different kinetic states obtained with the EFR. The size of the error bars is comparable to the discrepancy between the free-energy predictions using the NN model with the UO set of parameters16,17 and unzipping data18. To evaluate the free-energy branches of the different states (Fig. 4c) we could repeat the experiment for different final values of λ and measure the corresponding ΔG_{NB}(λ), B = N, I, I' or U. For simplicity we use an extended version of the Jarzynski equality obtained by multiplying the EFR by the reversed work distribution and integrating over the work (Supplementary Section S8),

$$\Delta G_{AB} = -k_B T \log \left( \frac{\phi_{AB}}{\phi_{BA}} \right) - k_B T \log \left( e^{-W/(k_B T)} \right)$$

Equation (2) requires only data from the F process and we apply it to pulling experiments recorded by setting extreme values of λ (light curves in Fig. 4a). Similar to the Jarzynski estimator2, the extended version of the Jarzynski equality is strongly biased24.
estimates the magnitude of the bias we took the difference between the free energy $\Delta G_{\text{UB}}$ obtained using equation (2) and the one obtained with the acceptance ratio method in pulling experiments where kinetic states are partially equilibrated at $\lambda_1$ (dark curves in Fig. 4a). Therefore, from the free-energy branches obtained using equation (2) we subtracted this estimated bias for each state (we assumed it to be equal for all values of $\lambda_1$, Supplementary Section S8). In contrast to I1, kinetic intermediates found in I2 become the most stable states in a given range of $\lambda$ (Fig. 4c). For low values of $\lambda$, stability is determined by N, and as $\lambda$ increases stability shifts to $\Gamma_1$, $\Gamma'$ and finally to U.

Hairpin M2 can fold into one native structure (N) and two misfolded structures (M', M") following alternative folding pathways (Fig. 5a and Supplementary Section S1). Whereas it is easy to identify trajectories that fold into N (red/blue FDCs in Fig. 5a left, ~50% of trajectories), distinguishing trajectories that misfold into M' or M" is not straightforward. Careful inspection reveals two different patterns of unfolding curves that start at a misfolded state: either the molecule unfolds quasi-reversibly without intermediates (purple FDC in Fig. 5a middle, ~30% of trajectories), or it folds back to N before it unfolds (cyan FDC in Fig. 5a right, ~20% of trajectories). We interpret the former as trajectories following the M' → U pathway and the latter as following the M" → N → U pathway (Supplementary Movie SVM2). This is supported by two facts (Supplementary Section S5).

First, M' consists of four small hairpins that confer low mechanical stability to the structure that gently unfolds under tension. Second, M" has a large stem in common with N (Fig. 5a, top) which is surrounded by two small hairpins with low mechanical stability. Once these two hairpins unfold around 9–10 pN, the force remains low enough for the molecule to fold back to N before unfolding. Combining equation (1), the partial work distributions (Fig. 5b) and handles and ssDNA elastic contributions leads to the free-energy values $\Delta G_{\text{NU}} = 94 \pm 2$, $\Delta G_{\text{MFU}} = 60 \pm 3$ and $\Delta G_{\text{MFU}} = 70 \pm 3 k_B T$, in agreement with theoretical predictions (Table 1). Figure 5c shows the reconstruction of the four thermodynamic branches relative to the full equilibrium free energy of the system, $\Delta G = -k_B T \log \sum_{A=N,M,M',U} e^{-\Delta G_{\text{AU}}/k_B T}$, by fixing $\lambda_1 = 230 \text{ nm}$ and varying $\lambda_0$ between 0 and 150 nm.

Summarizing, we have shown how the EFR can be used to extract free energies of non-equilibrium kinetic structures in DNA hairpins exhibiting intermediate and misfolded states. The method works accurately in far-from-equilibrium situations and when equilibrium experiments are insufficient to characterize non-native states. There are two main differences between the EFR in equation (1) and the Crooks relation:\ the partial work distributions and the prefactor $\phi_{\Delta A-B}^{\Delta A-B} / \phi_{\Delta A-B}^{\Delta A-B}$, which introduces the further correction $-k_B T \log (\phi_{\Delta A-B}^{\Delta A-B} / \phi_{\Delta A-B}^{\Delta A-B})$ into the Crooks estimation of the free-energy difference between kinetic states. The omission of such a correction yields wrong relative thermodynamic
in many molecular reactions, such as RNA, proteins and many kinetic states related to intermolecular binding, or transient non-equilibrium states that are essential in polymerization reactions (for example Adenosine-triphosphate or Adenosine-diphosphate bound states in motor proteins).

Methods

Molecular synthesis. The designed DNA molecules, linked to 29 bp double-stranded (ds)DNA handles, were synthesized as described in ref. 19. For the specific attachments to the DNA molecular construction we used streptavidin-coated polystyrene microspheres (1.87 μm, Spherotech) and protein G microspheres (3.0–3.4 μm; G. Kisker Gbr, Products for Biotechnologie) coated with anti-digoxigenin polyclonal antibodies (Roche Applied Science). Attachment to the anti-digoxigenin microspheres was achieved by first incubating the beads with the tether DNA. The second attachment was achieved in the fluidsics chamber and was accomplished by bringing a trapped anti-digoxigenin and an immobilized streptavidin microsphere close to each other.

Bennett acceptance ratio method. This method is used to estimate the free-energy difference ΔG_{M→U} between two states from non-equilibrium work measurements. Given a set of n_{F}(n_{R}) forward (reversed) work measurements W_{i}, it is shown in refs 21,22 that the solution u of the following transcendental equation:

\[ u = -\log \left( \frac{\phi_{\Lambda}^{\langle B \rangle}}{\phi_{\Lambda}^{\langle A \rangle}} \right) + z_{u}(u) - z_{s}(u) \]  

The main limitation of the method is the identification of kinetic states from the measured signal. In this regard, a combination of fluorescence techniques, such as fluorescence resonance energy transfer, with force measurements, and the application of advanced statistical methods (for example, hidden Markov models or Bayesian inference) might be very useful. Our methodology should find many applications that range from molecular biophysics to condensed matter physics. Any situation where equilibrium experiments are impractical should be treatable with different versions of equation (1). To start with, the method can be employed in measuring the free energies of kinetic structures that appear in many molecular reactions, such as RNA, proteins and many kinetic states related to intermolecular binding, or transient non-equilibrium states that are essential in polymerization reactions (for example Adenosine-triphosphate or Adenosine-diphosphate bound states in motor proteins).
Figure 5 | Hairpin M2, with two misfolded states. a, Patterns identified in the FDC with corresponding unfolding/folding pathways. N and M" share a piece of hairpin in their folded conformation (orange). Left: unfolding (red) and folding (blue) FDCs for state N show a force rip (∼2 pN) around 15 pN. Middle: unfolding (purple) and folding (green) FDCs for state M' show no hysteresis. Right: folding (grey) FDCs for state M" are identical to the ones measured for M', whereas unfolding (cyan) FDCs show a rescue to the N state. Six molecules were pulled, obtaining a minimum of 40 cycles and a maximum of 100 at 60 nm s⁻¹. b, Partial work histograms for work values measured between λ₁ = 0, where A = N (red, top panel), M' (blue, middle panel) or M" (green, bottom panel), and λ₁ = 230 nm, where B = U. Dark colours refer to unfolding and light colours to folding work distributions. c, Free-energy branches of states N (red), M' (blue), M" (green) and U (purple) measured relative to the full free energy of the system, ΔG = −k_BT log ∑_{A=N,M',M",U} P_A / P_B. Error bars in b,c indicate the standard statistical computed over 100 cycles for a given molecule. These were obtained using the bootstrap method.

where

\[ z_u(u) = \log \frac{1}{N_u} \sum_{i=1}^{N_u} \left( \frac{e^{-W_i/uBT}}{1 + e^{-W_i/uBT}} \right) \]

\[ z_l(u) = \log \frac{1}{N_l} \sum_{i=1}^{N_l} \left( \frac{1}{1 + e^{-W_i/u_BT}} \right) \]

minimizes the statistical variance of the free-energy estimation for \( u = \Delta G_{AB} \).

The right-hand side of equation (3) is expected to provide a constant function near the solution of the transcendental equation, as shown in Figs 2c and 3c for each branch.

**Free-energy recovery at zero force.** The solution of the Bennett acceptance ratio method gives the free-energy difference between state B at \( \lambda_1 \) and state A at \( \lambda_0 \). To recover the free energy at zero force of each structure with respect to the random coil state, \( \Delta G_{AB} \), we need to subtract the free energy of stretching the ssDNA, \( W_{\text{ssDNA}} \), the free energy of orientation of the hairpins stem, \( W_{\text{stem}} \), and the reversible work performed to stretch the handles and displace the bead in the optical trap, \( W_{\text{trap}} \):

\[ \Delta G_{AB} = \Delta G_{AB} - W_{\text{ssDNA}} - W_{\text{stem}} - W_{\text{trap}} \]  

To estimate the free energy of the handles and the bead by integrating a linear FDC along the folded branch from the minimum force at \( \lambda_1 \) to the maximum force at \( \lambda_1 \), that is, \( W_{\text{trap}} = (f_{\text{min}} - f_{\text{max}}) / k_BT \) where \( k_BT \) is the slope of the FDC (ref. 26).

**Free-energy prediction.** To obtain the most stable structure of the DNA molecules under study we use the mfold web server23. To predict the free energy of formation of each structure we use the NN model (Supplementary Section S1). The base-pairing free energies have been derived in thermal denaturation experiments16 and independently verified in single-molecule experiments16.

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Author contributions
I.J. and F.R. designed the experiment. A.A. made the measurements. A.A. and A.M. analysed the data. All authors wrote the paper.

Additional information
Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to F.R.

Competing financial interests
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