

# Quantitative and Predictive Folding Models from Limited Single-Molecule Data Using Simulation-Based Inference

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Single-molecule force spectroscopy resolves folding dynamics one molecule at a time, but extracting quantitative free-energy landscapes typically requires extensive datasets and careful instrument calibration to disentangle the molecule from linker and apparatus artifacts. We introduce a simulation-based inference framework that combines physics-based modeling with deep learning to recover the Bayesian posterior of a folding model directly from a single short trajectory. A 2-s constant-force measurement of a DNA hairpin is sufficient to reconstruct the folding landscape, matching deconvolution baselines that require 20–100 times more data and eliminating separate linker or instrument characterization. The same approach recovers four metastable states of a riboswitch aptamer from a single 5-s trajectory. Every parameter, including diffusion coefficients and linker stiffness, is included in the calibrated posterior. The current implementation assumes one-dimensional Markovian dynamics, but more complex models can be substituted within the same framework, opening single-molecule force spectroscopy to high-throughput parallel experiments and to systems where extensive data collection is impractical.

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Biomolecular folding is a fundamental process in which proteins, nucleic acids, and other biopolymers adopt three-dimensional structures essential for their function [1]. Understanding folding mechanisms is crucial to elucidating the principles of biomolecular self-organization and its connection to diseases. The folding dynamics arise from a complex interplay between many intermolecular forces, often modeled as a diffusive process on a rugged free-energy landscape [2]. Characterizing the folding free-energy landscape provides a complete thermodynamic picture of the folding process, providing insights into mechanisms and kinetics. However, experimentally obtaining free-energy landscapes remains challenging due to limitations in spatial and temporal resolution [3].

Single-molecule force spectroscopy (SMFS) probes the folding dynamics of individual biomolecules held under tension [4,5]. These measurements enable the

reconstruction of free-energy landscapes along a one-dimensional reaction coordinate, usually the molecular extension [6–10]. Such reconstructions have been achieved using constant-force [11], constant-position [12,13], and nonequilibrium measurements [14].

However, SMFS measurements are indirect: the biomolecule is connected to a large pulling device through long, flexible linkers, so the measured extension reflects a convolution of molecular dynamics, linker fluctuations, and the instrument response [15,16]. Measurement noise and the inherent stochasticity of single-molecule trajectories further complicate the estimation of the underlying free-energy landscape. In principle, the linker contribution can be removed by deconvolution [7,11,17]. In practice, this requires a large amount of data and a very precise characterization of the linker, which is technically challenging and labor-intensive. Consequently, accurate free-energy reconstructions exist only for a handful of molecules.

An alternative approach to overcome these problems is to reconstruct the free-energy landscape by fitting a dynamical model  $\mathcal{M}(\theta)$  to the observed trajectory  $\mathbf{q}_{[1:N]}$ , a vector containing  $N$  measured extensions sampled at equal time intervals. The parameters  $\theta$  determine the

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landscape and must be inferred from the observed trajectory [18]. Bayesian inference provides a systematic statistical framework to determine the posterior distribution over  $\theta$  that is most consistent with  $\mathbf{q}_{[1:N]}$ ,

$$p(\theta|\mathbf{q}_{[1:N]}) = \frac{p(\mathbf{q}_{[1:N]}|\theta)p(\theta)}{p(\mathbf{q}_{[1:N]})}. \quad (1)$$

Here,  $p(\theta)$  is the prior, encoding prior knowledge,  $p(\mathbf{q}_{[1:N]}|\theta)$  is the likelihood of observing the trajectory  $\mathbf{q}_{[1:N]}$  given  $\theta$ , and  $p(\mathbf{q}_{[1:N]})$  is a normalization factor.

Typical inference algorithms maximize the likelihood with respect to the parameters  $\theta$ . However, evaluating the likelihood for partially observable dynamical models is very challenging because it is a marginalized likelihood over all possible “latent” trajectories of the actual molecular extension  $\mathbf{x}_{[1:N]}$  [19,20],

$$p(\mathbf{q}_{[1:N]}|\theta) = \int \mathcal{D}\mathbf{x}_{[1:N]} p(\mathbf{q}_{[1:N]}, \mathbf{x}_{[1:N]}|\theta). \quad (2)$$

An emerging approach to tackle this problem is simulation-based inference (SBI) [18,20,21], which integrates physics-based models with probabilistic deep learning to learn a surrogate model of the posterior [22], likelihood [23], or likelihood ratio [24]. The core idea is to use a simulator to generate synthetic measurements for specific parameters  $\theta$ . Using deep neural networks, we can learn a surrogate model of the posterior distribution from the simulated data, providing a probabilistic mapping between parameters and data. SBI has shown success across various fields, from astrophysics [25,26] to neuroscience [27,28], particularly for models with complex likelihoods [29].

In this Letter, we showcase how to leverage simulation-based inference to learn quantitative folding models from single-molecule force spectroscopy experiments by learning the Bayesian posterior from synthetic data. This framework yields accurate uncertainty estimates and quantitative predictive models, providing an efficient and robust approach for analyzing SMFS data. We applied it to the well-characterized 30R50/T4 DNA hairpin as a model system [16,30–32], and showed that it can reconstruct the hairpin’s free-energy landscape even from limited experimental data. We further demonstrated the generality of the approach by applying it to the multistate folding of a riboswitch aptamer, highlighting its applicability to more complex systems.

Single nucleic acid hairpins fold into simple structures, offering a well-defined and biologically relevant experimental framework for investigating folding mechanisms [33–35]. The end-to-end distance of the DNA serves as a convenient reaction coordinate for the folding reaction, along which the dynamics are well characterized by one-dimensional Brownian diffusion on an effective free-energy landscape [30,36].

We applied our inference framework [18] to learn a quantitative folding model from constant-force measurements of a 30R50/T4 DNA hairpin. This particular hairpin has been extensively studied, with measurements of the folding dynamics spanning from microseconds to minutes and folding energy landscape reconstructions using various methods and experimental setups [7,14,30,37,38].

The harmonic-spring model  $\mathcal{M}(\theta)$  provides a well-established framework for describing single-molecule force spectroscopy experiments [10,15,19]. It describes the coupled dynamics of the biomolecule and the measuring apparatus as a diffusive process on a two-dimensional free-energy surface  $G(q, x)$ . Here,  $q$  is the measured extension, including both the linker and the molecule, while  $x$  is the (hidden) molecular extension. The free-energy surface decomposes as  $G(q, x) = G_0(x) + 1/2 \cdot k_l(q - x)^2$ , where  $G_0(x)$  is the intrinsic folding free-energy landscape of interest. The second term describes the linker coupling to the apparatus. We assume anisotropic Brownian dynamics on this energy landscape, with diffusion coefficient  $D_x$  for the molecule and  $D_q$  for the apparatus. The molecular free-energy profile  $G_0(x)$  is modeled using cubic spline interpolation. The model parameters  $\theta = \{D_q/D_x, k_l, G_0(x_1), G_0(x_2), \dots, G_0(x_{N_{\text{node}}})\}$  are the ratio of diffusion coefficients  $D_q/D_x$ , the linker stiffness  $k_l$ , and  $N_{\text{node}}$  positions of the spline nodes  $G_0(x_i)$  to characterize the free-energy profile  $G_0(x)$ . Inferring  $\theta$  from a measured trajectory  $\mathbf{q}_{[1:N]}$  fully characterizes biomolecular folding as diffusion on the one-dimensional landscape  $G_0(x)$ .

Direct computation of the likelihood [Eq. (2)] is challenging because it requires integrating over all hidden molecular trajectories  $\mathbf{x}_{[1:N]}$ . In contrast, simulating a measured trajectory  $\mathbf{q}_{[1:N]} \sim p(\mathbf{q}_{[1:N]}|\theta)$  is straightforward. SBI offers a general solution to perform Bayesian inference with intractable likelihoods when forward simulations are feasible. SBI starts by generating prior samples  $\theta_i \sim p(\theta)$ , which are used to simulate synthetic trajectories  $\mathbf{q}_{[1:N],i} \sim \mathcal{M}(\theta_i)$  [Fig. 1, upper panel]. The simulated dataset  $\mathcal{D} = \{(\mathbf{q}_{[1:N],i}, \theta_i)\}_{i=1}^M$  is then used to train a neural network-based density estimator  $f_\psi(\theta|\mathbf{q}_{[1:N]})$  of parameters  $\psi$  to approximate the Bayesian posterior [Fig. 1, center panel]. The neural network parameters  $\psi$  are optimized by minimizing the loss function  $\mathcal{L}(\psi) = -1/M \sum_i^M \log f_\psi(\theta_i|\mathbf{q}_{[1:N],i})$  on  $\mathcal{D}$ . After training, the posterior for an experimental trajectory is obtained as  $p(\theta|\mathbf{q}_{[1:N]}^{\text{exp}}) \approx f_\psi(\theta|\mathbf{q}_{[1:N]}^{\text{exp}})$ , enabling inference of the folding parameters [Fig. 1, right panel].

We analyzed data from the 30R50/T4 DNA hairpin [Fig. 2(a) cartoon]. Our analysis used a short 2-s constant-force measurement  $\mathbf{q}_{[1:N]}^{\text{exp}}$  [Fig. 2(a)] containing seven transitions between the folded and unfolded states. From this trajectory, we inferred the folding free-energy profile

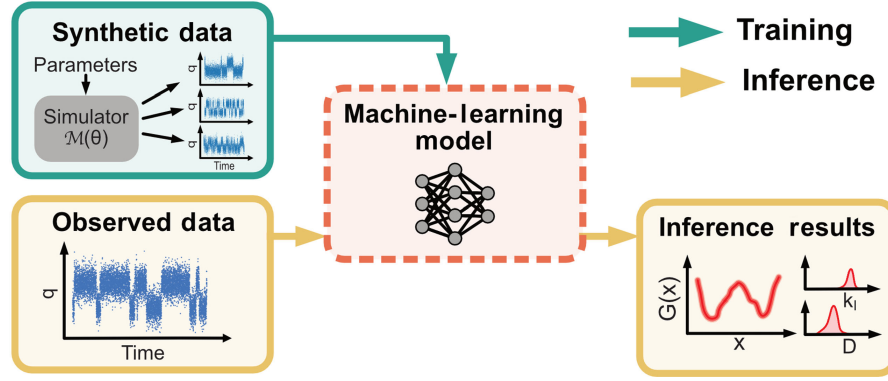


FIG. 1. Framework for analyzing single-molecule force spectroscopy data using simulation-based inference. The process begins by generating simulated trajectories via a physics-based simulator (top left). These trajectories are used to train a machine-learning model to establish probabilistic relationships between model parameters and synthetic data (middle). The trained model is then evaluated using experimental data (lower left), producing a distribution of model parameters that are compatible with the experimental observations (lower right).

[Fig. 2(b)] (see End Matter). The red line represents the best-fitting profile, obtained from the maximum *a posteriori* (MAP) parameters  $\theta_{\text{MAP}}^{\text{exp}}$ . The profile shows a barrier height of approximately  $9.9 k_B T$ . The thin blue lines show free-energy profiles generated from posterior samples  $\theta^{\text{exp}}$  within the 68% confidence interval, quantifying the uncertainty of the estimate [Fig. S2 in Supplemental Material (SM) [39]]. Sampling parameters consistent with  $q_{[1:N]}^{\text{exp}}$  from the posterior yield a median barrier of  $9.5 \pm 1.3 k_B T$  (Fig. S3 [39]).

We validated our results by comparing our inferred free-energy landscape with one obtained via deconvolution [Fig. 2(b) black dashed line]. Deconvolution is an established method that reconstructs the free-energy landscape by iteratively removing measurement noise from the experimental data (see SM [39]) [7]. Using measurements of the biomolecule-linker system  $G(q)$  and the linker only  $V(q - x)$ , deconvolution aims to find  $G_0(x)$ , that, when convolved with  $V(q - x)$ , reproduces  $G(q)$ . While both approaches yield closely matching profiles, confirming the reliability of our inference, deconvolution requires substantially more experimental data—approximately 20–100 times as many folding and unfolding transitions. This makes deconvolution more labor-intensive [7] and prone to errors arising from instrumental drift. Furthermore, deconvolution requires a separate linker characterization, where inaccuracies can introduce errors in the resulting landscape.

Additionally, we demonstrated that our estimated uncertainties are accurate by applying our inference framework to 20 independent 2-s measurements  $\{q_{[1:N],i}^{\text{exp}}\}_{i=1}^{20}$  of the same DNA hairpin molecule [Fig. 2(c)], obtaining statistically consistent free-energy landscapes across all measurements. The spread of these independently inferred profiles [red lines in Fig. 2(c)] falls within the uncertainty bounds [blue lines in Fig. 2(b)] determined from analyzing

a single trajectory, indicating that the error model of the posterior is well-calibrated.

The model we fitted to the experimental data  $q_{[1:N]}^{\text{exp}}$  also provides estimates for the ratio of diffusion coefficients  $D_q/D_x$  and linker stiffness  $k_l$ . Figure 3 reports the marginal posterior distributions for 20 independent experimental trajectories. The posteriors overlap across measurements, but their spread is larger than expected, indicating model misspecification (see SM [39]).

To further assess our model’s accuracy and predictive power, we simulated trajectories using the best-fitting parameters, i.e.,  $q_{[1:N],i} \sim p(q_{[1:N]} | \theta_{\text{MAP},1}^{\text{exp}})$ . We then compared these simulated trajectories to the experimental one [Fig. 4(a)]. Visual comparison shows excellent agreement between the simulated and experimental trajectories. To quantify this, we performed a total of 20 simulations  $\{q_{[1:N],i}^{\text{sim}}\}_{i=1}^{20}$  using the best-fitting parameters  $\theta_{\text{MAP},1}^{\text{exp}}$ . First, we compared the thermodynamics of the observed trajectories by inspecting the potential of mean force (PMF)  $G(q)$ . We computed the average PMF  $G(q)$  by combining all trajectories and binning them along  $q$ . The simulated and experimental PMFs align well in the folded and unfolded states, though the simulated trajectories exhibit a slightly higher barrier in the transition region [Figs. 4(b) and S4 [39]].

We also compared the observed kinetics by analyzing folding and unfolding transitions (see SM [39]). The experimental transition rate of  $2.8 \pm 0.3 \text{ s}^{-1}$  is close to the simulated rate of  $2.2 \pm 0.2 \text{ s}^{-1}$ .

Finally, we compared the autocorrelation functions of the measured extension in the folded and unfolded states [Fig. 4(c)]. While the autocorrelation functions align well in the folded state (upper panel), the experimental trajectories in the unfolded state exhibit a decay that is not single-exponential, behavior that is not captured by our simulations (lower panel). This discrepancy is consistent with

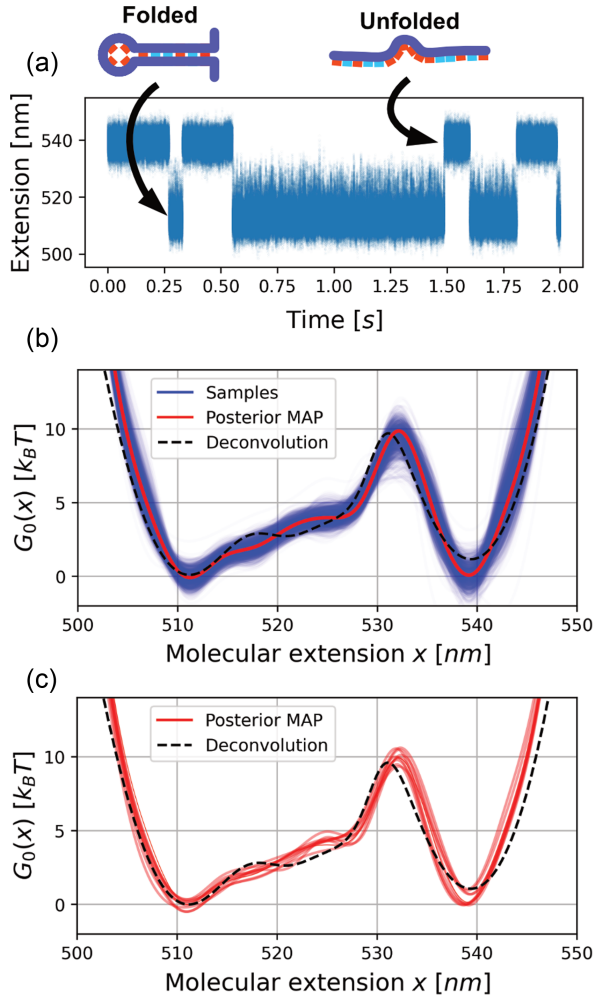


FIG. 2. Free-energy profile reconstruction. (a) Experimental time series used for inference. (b) Reconstructed free-energy profile. Best estimate  $\theta_{\text{MAP}}^{\text{exp}}$  (Maximum *a posteriori*, MAP) in red, and posterior samples covering a 68% confidence interval as blue thin lines. The dashed black line indicates the estimate using deconvolution. (c) Best free-energy profile (MAP) estimate for 20 independent experimental time series.

previously reported memory effects for the 30R50/T4 hairpin [46]. This suggests that a Markovian diffusion model may not fully describe the complex dynamics of an unfolded DNA hairpin [47–50].

To show that the same analysis framework can be applied to molecules with more complex folding, including multiple intermediate states and formation of tertiary contacts, we analyzed the folding of an RNA riboswitch aptamer. We used a 5-s constant-force measurement  $q_{[1:N]}^{\text{exp}}$  of the *add* riboswitch aptamer, whose folding pathway involves five states, where in this particular trajectory four out of five states are explored [51]. The riboswitch folds by sequentially forming two hairpins that then form a ligand-binding pocket organized by tertiary contacts [Fig. 5(a)]. We used

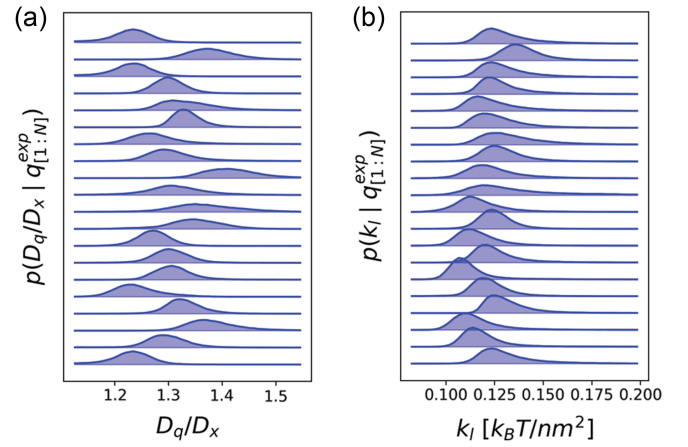


FIG. 3. Diffusion coefficients and linker stiffness estimates. Posteriors obtained using 20 independent experimental time series, quantifying the inference on (a) the ratio of diffusion coefficients  $D_q/D_x$ , and (b) the linker stiffness  $k_l$ . Posterior marginals were obtained by sampling and histogramming. All marginals are normalized to unit area. A constant vertical offset is applied between marginals to aid visualization.

sequential neural posterior estimation on  $q_{[1:N]}^{\text{exp}}$  to infer a folding model of the riboswitch (see SM [39]). The folding free-energy landscape shows four distinct states, each corresponding to one of the folding intermediates we expected to observe [Fig. 5(b)]. The landscape is consistent with results from previous single-molecule studies [51], identifying positions and energies of the potential wells and barriers that match previous analyses within error [Fig. 5(b), black]. Finally, predictive checks validate the fitted model, as trajectories  $q_{[1:N]}$  simulated with  $\theta_{\text{MAP}}^{\text{exp}}$  are consistent with the experimental measurement  $q_{[1:N]}^{\text{exp}}$ . The potentials of mean force  $G(q)$ , computed from the simulated (blue) and experimental trajectory (red) also show excellent agreement [Fig. 5(c)].

Our simulation-based inference framework reconstructs free-energy landscapes that match those of established deconvolution methods with 20–100 times less data and no separate instrument calibration, jointly infers diffusion coefficients and linker stiffness, and assigns calibrated posteriors to every parameter. The model reproduces the experimental data; the minor discrepancies that remain are not a finite-data effect, since varying the trajectory length (Fig. S5 [39]) produces consistent landscapes. Inference also does not require single long trajectories: eight independent 0.125-s segments (1-s total) recover the same landscape as a full 2-s trajectory (Fig. S11 [39]). The framework is therefore directly applicable to high-throughput parallel SMFS, where many short per-molecule trajectories are the natural output.

The residual discrepancies between the model and the data therefore likely arise from other sources, such as

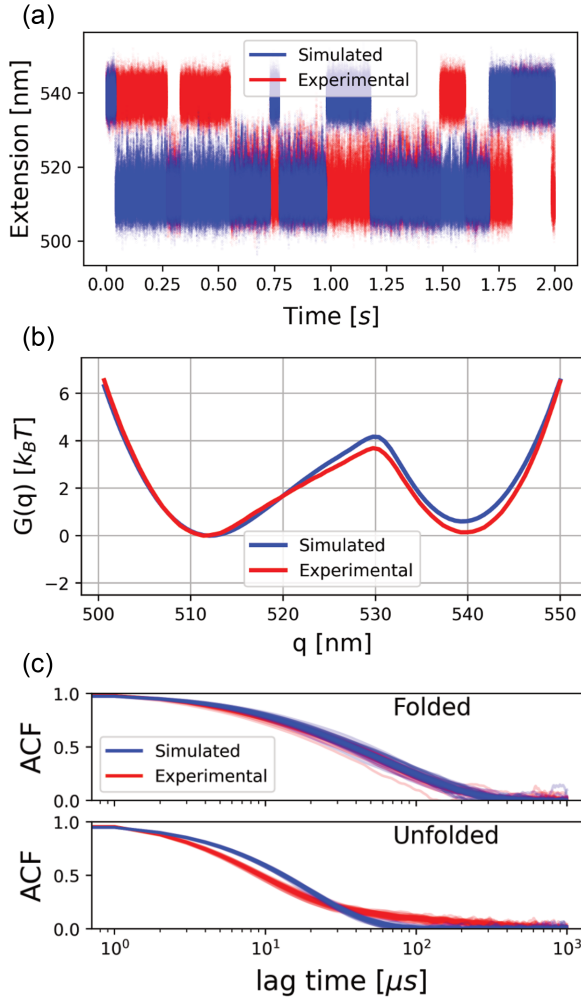


FIG. 4. Predictive checks using simulations with best-fitting parameters  $\theta_{\text{MAP}}^{\text{exp}}$ . (a) Trajectory  $q_{[1:N],1}$  simulated with  $\theta_{\text{MAP},1}^{\text{exp}}$  (blue) compared to experimental trajectory  $q_{[1:N],1}^{\text{exp}}$  used to make the inference (red). (b) The potential of mean force estimated from 20 synthetic trajectories  $q_{[1:N],i}$  (blue) simulated with  $\theta_{\text{MAP},1}^{\text{exp}}$  compared to the potential of mean force obtained from the experimental trajectories (red). (c) Autocorrelation functions for segments of the trajectories in the folded and unfolded states, respectively, comparing experimental  $q_{[1:N],i}^{\text{exp}}$  (red) and simulated trajectories  $q_{[1:N],i}$  (blue).

non-Markovian dynamics or position-dependent diffusion in the experimental data [46,52]. Such deviations suggest the need for a more complex model to capture the underlying folding dynamics more fully. Despite the simplicity of the model used in our analysis, our approach was still successful when extended from a simple two-state hairpin to a more complex structure featuring multiple intermediates and tertiary contacts.

Two extensions could improve predictive accuracy. Memory effects can be incorporated through more complex (e.g., generalized-Langevin) dynamics at the cost of parameters that lack direct physical interpretation.

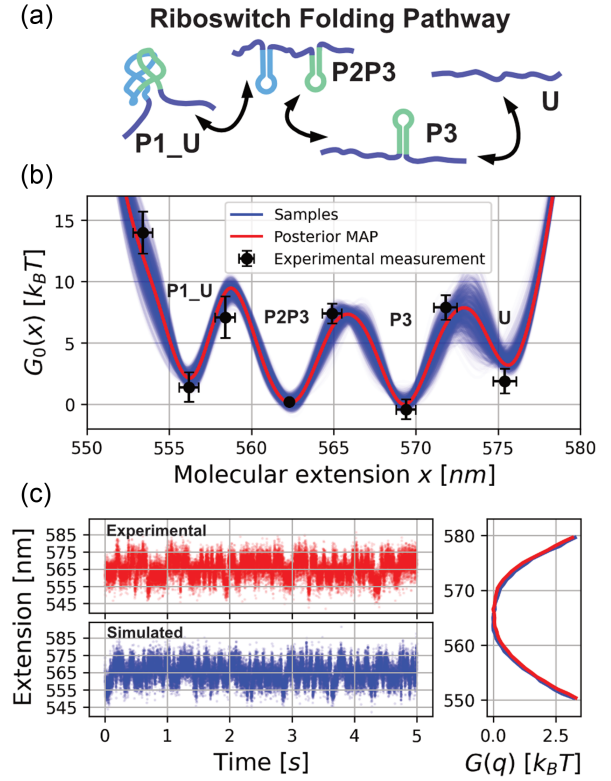


FIG. 5. Riboswitch folding. (a) Schematic illustration of the folding pathway observed during the constant-force experiment. Beginning from the unfolded state U, two hairpins, P2 and P3, are formed sequentially and subsequently establish a tertiary contact, P1\_U. (b) Reconstructed free-energy profile. Best estimate  $\theta_{\text{MAP}}^{\text{exp}}$  shown in red, and 68% confidence interval shown in blue. Positions and energies of the potential wells and barriers (black) previously deduced from experiments [51], measured relative to state P2P3. (c) Comparison between the experimental trajectory (red) and a simulated trajectory (blue) generated using  $\theta_{\text{MAP}}^{\text{exp}}$ . Potential of mean force computed from the experimental (red) and simulated trajectory (blue).

Alternatively, molecular dynamics can serve as the simulator, offering the highest fidelity at substantially higher computational cost.

By fitting a dynamical model directly to observed trajectories, SBI exploits temporal correlations and extracts statistically reliable folding models from limited datasets—critical for systems where extensive data collection is impractical. The framework extends naturally to other SMFS protocols, including constant-trap experiments.

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*Data availability*—The data that support the findings of this article are openly available [53]. Our code is available at [54] and is based on the sbi PYTHON package [55].

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## End Matter

*Sequential inference procedure*—We used sequential neural posterior estimation to approximate the posterior distribution  $p(\theta|q_{[1:N]}^{\text{exp}})$  for the experimental trajectory  $q_{[1:N]}^{\text{exp}}$ . The trained surrogate posterior can be evaluated on the experimental data, allowing parameters to be sampled from the posterior,  $\theta^{\text{exp}} \sim p(\theta|q_{[1:N]}^{\text{exp}})$ , to explore many parameter combinations consistent with the experiment. Alternatively, identifying the maximum *a posteriori* estimate,  $\theta_{\text{MAP}}^{\text{exp}} = \text{argmax}_{\theta} p(\theta|q_{[1:N]}^{\text{exp}})$ , yields

the best-fitting parameter set. Sequential neural posterior estimation approximates the posterior through iterative rounds of simulation and posterior density estimation. In each round, we carried out simulations with parameters drawn from the current posterior approximation. After 14 such rounds, each consisting of 2000 simulations, we obtained a surrogate posterior  $p(\theta|q_{[1:N]}^{\text{exp}})$  (Fig. S1 [39]) in approximately 11 h on a single Xeon Skylake Gold 6148 CPU.