Force-clamp experiments reveal the free energy profile and diffusion coefficient of the collapse of proteins

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Abstract

We present force-clamp data on the collapse of ubiquitin polyproteins in response to a quench in the force. These nonequilibrium trajectories are analyzed using a general method based on a diffusive assumption of the end-to-end length to reconstruct a downhill free energy profile at 5pN and an energy plateau at 10pN with a slow diffusion coefficient on the order of 100nm²/s⁻¹. The shape of the free energy and its linear scaling with the protein length give validity to a physical model for the collapse. However, the length independent diffusion coefficient suggests that internal rather than viscous friction dominates and thermal noise is needed to capture the variability in the measured times to collapse.

As measured the end-to-end length of proteins and RNA in response to force perturbations, single molecule experiments open a window into the complex dynamics of these molecules on their multi-dimensional energy potentials [1, 2, 3]. For example, a protein is unfolded by the application of a constant pulling force, while quenching the force to a low value triggers the hydrophobic collapse of the molecule [4]. This dynamical collapse has been modeled as a one-dimensional diffusion of the measured end-to-end length on a free energy profile in the case of protein monomers [5] and RNA molecules [6]. By contrast, dynamics in degrees of freedom hidden from the experiment were thought to govern the large diversity in the end-to-end length of trajectories visited by collapsing polyproteins [7]. Whether the experimental distribution of trajectories can be described by simple diffusion along the measured reaction coordinate or requires multiple dimensions remains an open question that requires novel analysis tools.

This question is non-trivial because the collapsing traces are out-of-equilibrium and standard techniques to reconstruct the free energy profile based on the
Jarzynski equality \[8\] or Crook's fluctuation theorem \[9\] are not applicable to the force quench experimental protocol. Indeed, these techniques rely on knowing the statistics of the work exerted on the system \[10,11,12,13\]. In force-clamp experiments this work is concentrated in the brief time it takes to quench the force (\(\sim 50\text{ms}\)), which would require a prohibitively large pool of data to ensure the statistical accuracy of the free energy estimator based on the Jarzynski equality \[14,15,16,17\]. A second difficulty is that the free energy alone is not sufficient to describe the dynamics of the collapse. If the collapse can be described by an overdamped Langevin equation for the end-to-end length of the protein \[5\], then a diffusion coefficient must be estimated besides the free energy \[18,19\].

Here we introduce an analysis method to reconstruct the free energy profile \[20\] directly from the collapse trajectories of ubiquitin polyproteins, assuming diffusive dynamics. By reconstructing the free energy for polypeptide chains with varying numbers of protein domains, we quantify to what extent the collapse mechanism is cooperative between the domains \[21\]. Moreover, the observation that increasing the quench force slows down the collapse process \[4,1\] is explained in terms of the shape of the reconstructed free energy landscape, which in turn tests the Bell model \[22\] with no adjustable parameters. We then present an extension to the approach that offers the first measurement of an effective diffusion coefficient of a collapsing polypeptide and tests its constancy along the measured reaction coordinate. Finally, we propose a microscopic origin for the observed collapse in terms of the worm-like chain and ‘expanding sausage’ models \[6\].

We use Atomic Force Microscopy (AFM) in the force-clamp mode to follow the unfolding and refolding trajectories of ubiquitin polyproteins under a constant stretching force, as shown in the example in Fig. 1. Exposing a mechanically stable protein to a high pulling force of 110pN leads to the stepwise unfolding and extension of each of the three protein domains in the polypeptide chain. Subsequently, quenching the force to a lower value of 10pN triggers the collapse of the whole protein from a fully extended state back to a collapsed state with the same end-to-end length as the folded protein. Previous experiments have shown that the final state of the collapse process does not lead to a mechanically stable folded protein, but a random compact globule that forms native contacts over time \[23\]. A second pull on the same protein at 110pN leads to a second unfolding, as shown in the trajectory. Here we analyze only those trajectories that exhibit a minimum of three steps of \(\sim 20\text{nm}\) in the initial staircase as a signature of the extension of individual ubiquitin domains upon unfolding, as well as a second staircase to signify refolding. The question is then to understand the mechanism of the collapse dynamics from many recordings (\(n_{\text{tot}} \sim 100\)) of these trajectories.

Theoretically, if we denote by \(x\) the end-to-end length, the overdamped Langevin equation reads
\[
\dot{x} = -\beta DG'(x) + \sqrt{2D} \eta(t)
\]
where \(\beta = 1/(k_B T)\), \(\eta(t)\) is a white-noise term accounting for thermal effects,
\(G(x)\) is the equilibrium free energy profile and \(D\) is the diffusion coefficient which we assume to be constant (this assumption is validated below). Both \(G(x)\) and \(D\), or the friction coefficient \(\gamma\), can be estimated from the collapsing traces using the techniques introduced in [20].

Let us consider the free energy first. The procedure to calculate \(G(x)\) from the collapsing traces is to cut out pieces of trajectories from the moment the force is quenched at the unfolded length, \(x_u\), until the moment they first reach the folded length at low force, \(x_f\). This allows us to estimate via binning a nonequilibrium stationary probability density \(\rho(x)\) of many collapsing trajectories, and relate \(G(x)\) to it for \(x \in [x_f, x_u]\) as follows:

\[
G(x) = -k_B T \ln \rho(x) - k_B T \rho'(x_f) \int_x^{x_u} dx'/\rho(x')
\]

where \(\rho'(x_f)\) denotes the derivative of \(\rho(x)\) estimated at \(x_f\). Note that this formula is different from the standard \(G(x) = -k_B T \ln \rho_e(x)\), where \(\rho_e(x)\) is the equilibrium probability density function. The nonequilibrium \(\rho(x)\) requires an additional term besides \(-k_B T \ln \rho(x)\) in (2) to relate it to \(G(x)\). This extra term corrects for the fact that \(\rho(x)\) is biased towards values of \(x\) that are closer to \(x_u\), where the trajectories are initiated by the protocol. For a detailed derivation of (2) we refer the reader to [20], where this formula is also compared to Bayesian inference methods [18, 24, 25]. Using Eq. (2) is advantageous because the chronological order in which the data is acquired does not play a role in the binning procedure, which implies that the time resolution of the instrument (\(\sim 5\)ms) has no impact on the resulting landscape.

Next we apply (2) to analyze force-clamp trajectories, such as the one shown in Figure 1. Since the length of the polyprotein chain and the polypeptide linker to the surface vary from one experiment to the next, we compare all trajectories in terms of the total length of the collapse \(L_{tot} = x_u - x_f\). We find that \(L_{tot}\) clusters in increments of a monomer ubiquitin length of \(\sim 20\)nm with a standard deviation of \(\sim 6\)nm. We therefore group the clusters of similar collapse lengths and estimate the number of domains in the polyprotein chain as \(N_d = L_{tot}/20\)nm to the nearest integer. Setting the lowest value of \(L_{tot}\) within a group of a given \(N_d\) to be \(x_u\) at time zero and \(x_f\) to \(4\)nm [26] as the folded length of the protein from the protein data bank, leads to the alignments of trajectories shown in Figs. 2A and 2B for the 10pN and 5pN force quench, respectively, in the group of \(N_d = 3\). Analyzing trajectories in groups segregated by \(N_d\), we measure the non-equilibrium distribution \(\rho(x)\) of the end-to-end length for each \(N_d\), as shown in the insets. At a quench force of 10pN, the extended polypeptides often plateau at \(\sim 70\%\) of the contour length before their final collapse. Lowering the quench force to 5pN reveals faster collapse trajectories that visit all end-to-end lengths with a similar probability.

Using the observed distributions, we then obtain \(G_{N_d}(x)\), the free energy of a polyprotein of \(N_d\) units, and collapse these different profiles onto one another.
using the rescaling

\[ G(x + x_f) \equiv G_{N_d=1}(x + x_f) = \frac{1}{N_d} G_{N_d}(N_d(x + x_f)) \] (3)

This cooperativity between the domains is inconsistent with previously proposed models for the stochastic refolding of individual domains [27] or the aggregation of the unfolded domains [28]. Instead, our result in (3) suggests a global collapse of the polypeptide chain due to the attraction between hydrophobic residues that do not directly lead to folding [23, 29, 30].

The shape of the resulting free energy profile \( G(x) \) per ubiquitin monomer in Fig. 4A at 10pN is interesting because of the absence of a barrier: the experimental collapse corresponds to a diffusive slide on a plateau in the free energy that accelerates as the end-to-end length reaches a value \( \sim 5\text{nm} \) away from \( x_f \). Lowering the force to 5pN eliminates the plateau landscape and promotes a downhill collapse that is limited by friction alone, which is roughly consistent with the prediction of the tilt by the Bell model, also shown in Fig. 4A. Similar features of ubiquitin monomer trajectories under a quench force of 10pN were interpreted in terms of a physical model that predicts a free energy profile with a barrier of \( 2.5k_BT \) [5]. Since tilting the profiles in Fig. 4A by the Bell model [22] to just 13pN leads to a barrier to collapse of the same height, this small difference in the quench force could explain the observed change in the profile. However, the functional form of the landscape proposed in [5] does not fit the free energy profiles accurately due to its propensity to form barriers over a wide range of quench forces.

A better fit is achieved using the physical model proposed for the collapse of RNA molecules [6], which is based on the sum of the entropic worm-like chain model, the work done on the protein and the enthalpic ‘expanding sausage’ model for polypeptide collapse [31]:

\[ G(x) = \frac{2k_BT}{\xi^2} \sqrt{\pi\Omega(x-x_f)} - F(x-x_f) + k_BT \frac{L_c}{l_p} \int_{x-x_f}^{x} \left( \frac{1}{4(1-y)^2} - \frac{1}{4} + y \right) dy \] (4)

Here \( F \) is the applied force, \( L_c \) and \( l_p \) are the contour and persistence lengths of the extended protein, respectively, \( \Omega \) is the volume of the sausage, and \( \xi \) is the size of a globule inside the sausage [31]. The adjustable parameters in Eq. (4) are \( l_p, L_c, \) and the ratio \( \sqrt{\Omega}/\xi^2 \). Fits to \( G(x) \) in Fig. 4A give \( L_c = 26\text{nm} \), predicted by the size of a ubiquitin monomer (76 residues \( \times 0.36 = 27.4\text{nm} \)) [26], \( l_p = 0.82\text{nm} \) at 5pN and 1.45nm at 10pN, in agreement with chain stiffening along the backbone due to intramolecular interactions [6]. To obtain the values of \( \Omega \) and \( \xi \) from their ratio given by the fit, we assume that the size of the individual monomers in the sausage is \( l_p \). This implies that the number \( N \) of these monomers must be \( N = L_c/l_p \). Following de Gennes’ argument, we then set \( \xi = l_p \sqrt{\Omega} \) and \( \Omega = L_c \pi \xi^2 = L_c \pi l_p^2 g \), where \( g \) is the number of monomers inside each globule and becomes the fit parameter that replaces the ratio. Fits
to $G(x)$ in Fig. 1 thus yield $\xi = 2.6\text{nm}$ at 5pN and $2.7\text{nm}$ at 10pN, in rough agreement with the value $\xi = 2\text{nm}$ estimated for the hydrophobic collapse \[32\], and $\Omega = 203.62\text{nm}^3$ at 5pN and $373.90\text{nm}^3$ at 10pN. Note that the above argument does not affect the quality of the fits, simply it gives an interpretation of the parameters in Eq (4) that indicates that the microscopic packing of blobs inside the initial sausage is different for the two quench forces. Note also that the functional form of this free energy is consistent with the scaling with $N_d$ in Eq. (3) since the volume of a polyprotein with $N_d$ domains is $N_d\Omega$ and its contour length $N_dL_c$ while all the other parameters in Eq. (4) are unaffected by $N_d$. Altogether, these results give a quantitative validation of the physical model underlying the collapse.

The collapsing traces can also be used to calculate the diffusion coefficient $D(x)$ on the reconstructed landscape and thereby verify our assumption that it is constant, $D(x) \approx D$. The idea is to replace $x_f$ by any $x \in [x_f, x_u]$ in the procedure, i.e. cut the traces from $x_u$ till the first moment they reach $x$ and recalculate their non-equilibrium probability density $\rho$. The probability flux of these traces through the end-point $x$ can be expressed in two ways: it is given by $D(x)\rho'(x)$, and it can also be estimated directly as $1/\tau_c(x)$, where $\tau_c(x)$ is the average time it takes them to collapse from $x_u$ to $x$. Equating these two expressions and solving for $D(x)$ gives

$$D(x) = \frac{1}{\tau_c(x)\rho'(x)} \quad (5)$$

This estimator for $D(x)$ is new and it has the advantage over the standard one using quadratic variation of the trajectory \[18\] that it is insensitive to the time resolution of the instrument. Because of the small number of traces per $N_d$ per force ($\sim 15$), the estimate for $D(x)$ is accurate over the plateau regime in the end-to-end length in the data set at 10pN and not in the drift dominated parts of the landscape. The results obtained for polyproteins with different $N_d$ in Fig. 4B are in good agreement with each other, within the experimental error, and show that the diffusion coefficient is roughly constant as a function of $x$, consistent with the assumption made in Eq. (1). This is a surprising result because the ‘expanding sausage’ model predicts a $1/x$ scaling of $D(x)$ due to an increase in the viscous friction as the molecule collapses to a blob of a growing radius. By contrast, here the protein dynamics is governed by internal rather than solvent friction, which agrees with recent single molecule experiments that show an independent friction with the end-to-end length of a folding protein \[33\]. Notice also that the average value of $170\text{nm}^2/\text{s}$ is orders of magnitude smaller than the typical vibrational modes of a protein \[35\]. This indicates that the projection of all the degrees of freedom of the molecule onto a single reaction coordinate manifests itself as a very slow diffusion. It is likely that many local barriers in other degrees of freedom (associated with the formation of e.g. loops or helices at the same end-to-end length) can be mimicked by an effective diffusion constant.

To verify our results, we generate artificial traces using Eq. (1) with the estimated $G(x)$ and $D$ and show that they are in excellent agreement with
experimental traces in Fig. 2. In addition, the fact that traces generated using $D$ derived at 10pN reproduce the spread of times to collapse and the noise fluctuations in the experimental traces at 5pN suggests that $D$ does not change with the quench force. We estimate that $\sim 70\%$ of experimental trajectories are consistent with the 1-D diffusive model, while the outliers do not agree with the synthetic distribution of collapse times. Such trajectories have been observed previously [7, 23] and they highlight the importance of other degrees of freedom. Nevertheless, the simulated and the experimental average times to collapse $\tau_c$ agree very well at both quench forces and for all $N_d$, as shown in the inset in Fig. 4B. By contrast, a linear scaling with $N_d$ of a barrier-limited $G(x)$ [5] would lead to a much steeper dependence of $\tau_c$ with $N_d$, which is inconsistent with our and other published polyprotein data [1].

This general non-equilibrium method to analyse single molecule trajectories has allowed us to reconstruct free energy profiles, assess the dynamics along the measured reaction coordinate and thus postulate a physical model for the collapse of ubiquitin proteins. This technique paves the path for a mechanistic approach to many complex problems, such as protein folding.

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Figure 1: A typical force-clamp trajectory of the unfolding and refolding of a polyubiquitin chain with $N_d = 3$ domains. A second pull to 110pN reveals a staircase as a signature that the protein domains refold.
Figure 2: Collapsing trajectories are grouped by their total length ($N_d = 3$) and aligned at the time of the force quench to 10pN in (A) and 5pN in (B). The experimental trajectories are compared with those generated by simulations of diffusive dynamics on the reconstructed free energy profiles.
Figure 3: The nonequilibrium distribution $\rho(x)$ for each $N_d$ collected at a force quench of 10pN in (A) and 5pN in (B). The linear rescaling by $N_d$ is shown in the inset, which indicates a cooperative mechanism for the collapse.
Figure 4: (A) Experimental free energy profiles as a function of the end-to-end length, rescaled by $N_d$. (B) Diffusion coefficients derived from Eq. (5) at 10pN (solid lines) compare well with those estimated from the free energy reconstruction (dashed lines). The inset shows $\tau_c$ dependence on $N_d$ (squares), consistent with simulated data (circles).