Single-Molecule Unfolding Force Distributions Reveal a Funnel-Shaped Energy Landscape

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ABSTRACT The protein folding process is described as diffusion on a high-dimensional energy landscape. Experimental data showing details of the underlying energy surface are essential to understanding folding. So far in single-molecule mechanical unfolding experiments a simplified model assuming a force-independent transition state has been used to extract such information. Here we show that this so-called Bell model, although fitting well to force velocity data, fails to reproduce full unfolding force distributions. We show that by applying Kramers' diffusion model, we were able to reconstruct a detailed funnel-like curvature of the underlying energy landscape and establish full agreement with the data. We demonstrate that obtaining spatially resolved details of the unfolding energy landscape from mechanical single-molecule protein unfolding experiments requires models that go beyond the Bell model.

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In the ''new view'' of protein folding a funnel-shaped energy landscape steers the folding polypeptide chain toward its native folded conformation and thus facilitates the folding problem. This view has emerged from a host of bulk studies and theoretical work (1). Recently single-molecule mechanical techniques have offered the possibility to study the stabilizing forces in protein folding directly (2). Up to now all information about the energy landscape extracted from single-molecule mechanical unfolding experiments has been limited to a force-independent barrier position and an unloaded unfolding rate. Although in the field of receptor-ligand mechanics multiple barriers have been reported (3,4), for forced protein unfolding such effects have never been observed. Experimental limitations, like calibration errors and ill-defined loading rate conditions, have precluded a more detailed insight into the barrier shape. In this letter we demonstrate that the commonly used Bell model to analyze velocity-dependent unfolding force data fails to describe force distributions and yields a misleading view of the unfolding energy landscape.

We investigated unfolding force distributions at various pulling velocities of the Immunoglobulin-like domain 4 (ddFLN4) from the *Dictyostelium discoideum* F-actin cross-linker filamin (ddFLN) (see also *inset* in Fig. 1 *A*). We chose this domain because it offers the possibility to perform experiments under well-defined loading rate conditions in repetitive unfolding-refolding cycles with a truly single domain. The details of these double-jump experiments and the applied methods for obtaining force distributions with ddFLN4 have been described in detail elsewhere (5,6). It is important to note that for a detailed analysis of force distributions, well-defined loading rate conditions through a constant spacer length are essential. Moreover, it is crucial to ensure that no events get missed in the thermal noise floor.

There are two independent ways of assessing the underlying energy landscape in single-molecule mechanical unfolding experiments. Commonly, the most probable unfolding force is plotted versus the pulling velocity. Typically a logarithmic dependence is observed. Force versus velocity for ddFLN4 unfolding is plotted in Fig. 1 *A*. The data appear almost perfectly logarithmic. A strict logarithmic dependence has usually served as justification to apply the so-called Bell model, a simple two-state model with a triangular energy profile (Fig. 1 *B*) exhibiting a force-independent transition state position (7). According to this model, force modulates the observed unfolding rate following

$$k(F) = k_{\mathrm{off}}(0) \times \exp\left(\frac{F \cdot \Delta x}{k_{\mathrm{B}}\mathrm{T}}\right),$$

where $k_{\mathrm{off}}(0)$ denotes the unloaded unfolding rate, Δx the transition state position, and $k_{\mathrm{B}}\mathrm{T}$ the thermal energy (7). From this force-dependent unfolding rate the probability distributions of rupture forces as well as the most probable unfolding force as a function of the pulling velocity can now be calculated analytically even if the force is applied through a nonlinear polymer spacer chain ((8); also Supplementary Material). Our data for the most probable unfolding forces in Fig. 1 *A* agree perfectly with a fit to the Bell model (*dotted line* in Fig. 1 *A*) with $k_{\mathrm{off}}(0) = 0.35\ \mathrm{s}^{-1}$ and $\Delta x = 5$ Å.

However, when trying to fit the full probability distributions for the unfolding force at the various pulling velocities with the Bell model using the above parameters, a strong disagreement between data and Bell model becomes evident (Fig. 1 *C*). At low pulling velocities the distributions according to the Bell model are much wider, exhibiting a higher fraction of low force events than our data (between 0

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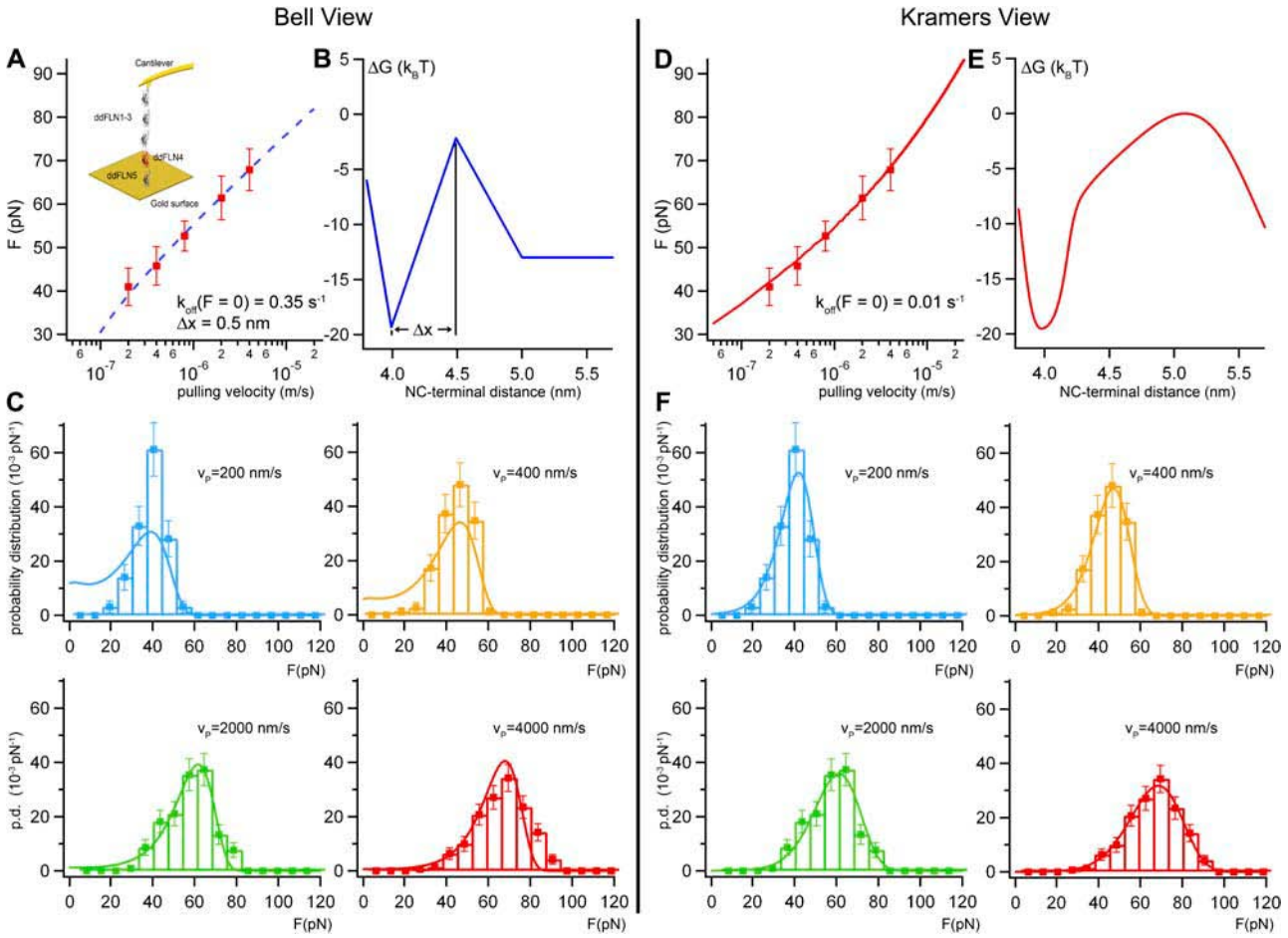

FIGURE 1 A comparison between the commonly used Bell view and Kramers' view for analysis of protein unfolding data. (*A*) The pulling velocity data of the native-state unfolding of ddFLN4 show a logarithmic behavior within the experimental error. The inset shows a schematic illustration of the experimental setup. (*B*) Schematic reconstruction of the energy landscape in the Bell view along the NC-terminal vector. (*C*) Normalized unfolding probability force distributions (histogram with statistical error) at four different pulling velocities in comparison with the theoretical distributions using the Bell model (*lines*). (*D*) Pulling velocity data modeled using Kramers' theory. (*E*) The reconstructed energy landscape shows detailed curvature along the unfolding/folding pathway. (*F*) The characteristic behavior of the experimental unfolding force distributions with increasing pulling velocity is reproduced well using the Kramers' model.

and 20 pN, ~25% of all events at 200 nm/s). At this point it is extremely important to be certain about the number of missed low-force events in the experiment. Because our experiment involves repeated unfolding-refolding cycles of a single ddFLN4 domain with well-studied refolding kinetics we can estimate the number of missed events to be <5% (for details see Supplementary Material). In contrast, the probability distribution at high pulling velocities (4000 nm/s) as calculated by the Bell model appears too narrow compared to the data. The changing width of the measured force distributions is a strong indication for a transition state position that moves with force.

How can we resolve the puzzling disagreement between an apparently logarithmic force-velocity behavior and yet a moving transition-state position? Obviously our data call for a more realistic model to describe unfolding force data. Previous theoretical studies have shown that the adaptation of Kramers' diffusion model to forced unbinding can give important information about the underlying energy landscape (9,10). Following Shillcock and Seifert (11) we calculated the mean first passage time for a particle in a given potential at increasing external forces according to:

$$\frac{1}{k(F)} = \frac{1}{D} \times \int_{x_{min}}^{x_{max}} \exp\left(\frac{-U_F(x)}{k_B T}\right) \times \left[\int_0^x \exp\left(\frac{U_F(x')}{k_B T}\right) dx'\right] dx,$$

where D represents the diffusion constant of the particle and $U_F(x)$ represents the energy profile along the pulling direction at force F (for details see Supplementary Material)

(11). We optimized the energy profile $U_F(x)$ shown in Fig. 1 *E* so that the full distributions were reproduced. It is interesting to note that although the transition state of the potential $U_F(x)$ is strongly force dependent the predicted force versus pulling velocity curve is still in perfect agreement with our data (Fig. 1 *D*). Although a slight curvature of the force-velocity curve can be observed over a large range of four decades, experimental data would still appear almost logarithmic. Moreover, without any additional fit parameter Kramers' theory also reproduces the full experimental probability distributions (Fig. 1 *F*).

The optimized energy profile has a funnel shape. Broad transition states have been observed in bulk studies, albeit along a very different reaction coordinate (12). Such a broad transition state has been suggested to actively steer the protein to its minimum from distant conformations.

Our results demonstrate that merely analyzing unfolding force versus velocity curves may yield an oversimplified and even sometimes misleading picture of the underlying energy landscape. It is also important that not only the transition state position as obtained by the Bell model is drastically smaller (5 vs. 11 Å) but also $k_{off}(0)$ is considerably too high (0.35 s^{-1} vs. 0.01 s^{-1}). This has important consequences when discussing $k_{off}(0)$ values extracted from force-velocity data analyzed using the Bell model.

To conclude, we could demonstrate that high-resolution recordings of full probability distributions are essential for characterizing in detail the energy landscape governing forced unfolding of proteins.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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