

Full distance-resolved folding energy landscape of one single protein molecule

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Kinetic bulk and single molecule folding experiments characterize barrier properties but the shape of folding landscapes between barrier top and native state is difficult to access. Here, we directly extract the full free energy landscape of a single molecule of the GCN4 leucine zipper using dual beam optical tweezers. To this end, we use deconvolution force spectroscopy to follow an individual molecule's trajectory with high temporal and spatial resolution. We find a heterogeneous energy landscape of the GCN4 leucine zipper domain. The energy profile is divided into two stable C-terminal heptad repeats and two less stable repeats at the N-terminus. Energies and transition barrier positions were confirmed by single molecule kinetic analysis. We anticipate that deconvolution sampling is a powerful tool for the model-free investigation of protein energy landscapes.

leucine zipper | force spectroscopy | optical tweezers | protein folding | deconvolution

The path of an unfolded protein toward its folded and functional conformation is entirely determined by its energy landscape (1). Experimental data often provide very limited view of these energy landscapes. Many proteins are classified as two-state folders, because barrier crossing is the rate limiting step and the subsequent motion toward the native state occurs extremely fast. Kinetic data hence lose almost all the information of the energy landscape on the native side of the transition state. A more detailed insight into the energy landscape of proteins consequently requires experimental data that go beyond classical kinetic assays (2). In recent years, single molecule mechanical methods have been successfully employed to study the energy landscape of biomolecules in increasing detail (3–7). Specifically for DNA, the analysis of equilibrium fluctuations upon application of mechanical load has provided sequence-resolved energy profiles of the full energy landscape (4). For proteins, such a detailed description has so far remained elusive.

The leucine zipper of the yeast transcriptional activator GCN4 is an ideal protein model system for studying real time folding/unfolding dynamics to obtain spatially resolved energy profiles. Because of its simple linear folding topology, the mechanically unzipped length can be directly linked to the amino acid position of the unzipping fork. The GCN4 zipper domain contains four heptad repeats forming a double-stranded α -helical coiled coil (8) and has been described as a two-state folder (9). Bulk folding studies have shown that folding of a cross-linked coiled coil is nucleated at the C-terminal end of the protein (10, 11). From there, zippering of the coiled coil proceeds toward the N-terminus. Activation energies and folding kinetics have been investigated extensively (9, 12, 13). Earlier single molecule mechanical experiments using atomic force microscopy have provided insight into the average unfolding forces of the zipper domain (14, 15). Limited force resolution in atomic force microscopy (AFM) experiments, however, has precluded the direct observation of folding/unfolding transitions in this system.

Here, we use single molecule force spectroscopy by optical tweezers (3) to directly measure the full free energy landscape of a GCN4 based leucine zipper. The experimental design is

sketched in Fig. 1A. We used a fusion construct consisting of a sequence of three identical GCN4-p1q domains (construct LZ26, see *Methods* and *SI Text*) (11, 14). This triple zipper domain construct offers the possibility to study the GCN4-p1q energy landscape with nucleation (C-terminal domain shown in blue) and simultaneously nucleation free (N-terminal domains shown in green and red) (15). The protein is clamped between two beads using DNA handles attached to N-terminal Cysteins (see *SI Text*) (3). One bead is moveable with respect to the other to control the tension applied to the protein.

Results and Discussion

In a first set of experiments we recorded force vs. extension traces at constant trap velocity. In Fig. 1B, four successive unzipping (Black) and rezipping (Blue) cycles pulled at 500 nm/s are shown. Unfolding of the LZ26 zipper results in a highly reproducible characteristic folding/unfolding pattern at forces between 8 and 15 pN (Fig. 1B, *Inset*). Upon force application, two intermediates (I_1 and I_2) can be observed. Starting from the fully folded state N, I_1 is populated in a smooth hump-like transition at equilibrium. Transition to I_2 occurs close to equilibrium; however, distinct flips of the molecule between the two intermediate states are resolved. If stretched further, a final transition occurs to the completely unfolded configuration U of the molecule. Upon reversal of the pulling process the molecule refolds, exhibiting a hysteresis at a pulling velocity of 500 nm/s. To relate the observed intermediate configurations to the sequence of the protein, we used a serial worm like chain model to fit the force vs. extension traces (see Table 1, *SI Text*, and Fig. S1). The positions of the intermediate states correspond well to the positions of asparagine residues in the sequence, which are known to destabilize the coiled coil (14, 16).

The low instrumental drift of our setup allowed observation of thousands of transitions of one molecule between different protein conformations held at defined pretensions (the force acting on intermediate I_1) at constant trap separation. A typical force vs. time trace is shown in Fig. 1C (*Upper*). Because trap separation but not force is kept constant, every length change of the protein is associated with a change in tension. A zoom into the long data trace (*Lower*) allows observation of equilibrium transitions between the unfolding intermediates I_1 and I_2 as well as the completely unfolded protein U (shown as red, green, and blue dashed lines). The lines are determined as maxima of Gaussian fits to the data. The red and green lines appear slightly closer than expected from the contour length increases. This discrepancy indicates a deviation from a quadratic shape of the underlying folding energy landscape.

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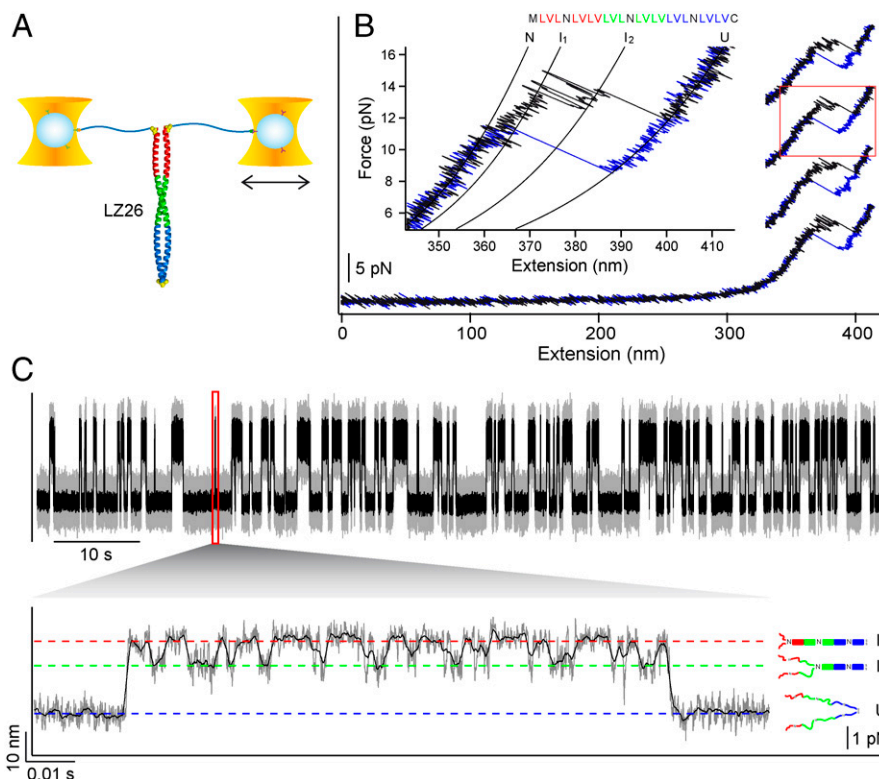


Fig. 1. Experimental setup and characteristic sample traces. (A) Cartoon depicting the experimental setup. The LZ26 coiled coil containing three GCN4 leucine zipper domains is attached to two beads via dsDNA handles. Individual zipper domains are coloured red, green, and blue. The two α -helical strands are cross-linked by cysteines at the C-terminus to avoid dissociation upon complete unfolding (see *SI Text*). (B) Force vs. extension traces of the LZ26 coiled coil. Four sequential unzipping (Black) and rezipping (Blue) cycles at 500 nm/s are shown (offset in force for clarity) (Inset) Magnification of the cycle marked by the red square. Unfolding from the native state (N) to the unfolded state (U) occurs via two resolved intermediates (I_1 and I_2). Lines are fits of a serial worm-like-chain model to the data (see Table 1 and *SI Text*). The letters specify amino acid residues in A and D positions of the coiled coil. (C) Force vs. time record of the LZ26 coiled coil held at a pretension of 14.1 pN (Upper) at constant trap separation. The magnification (Lower) of the region marked by the red square allows observation of transitions between I_1 (Red Dashed Line), I_2 (Green Dashed Line), and U (Blue Dashed Line). The structure of these conformations is sketched on the right. The y axis represents bead deflection from the trap center, which is linearly connected to the force acting on the molecule.

Increasing the separation between the two traps, and hence the pretension on the molecule, shifts the population probability from the folded to the unfolded state (Fig. 2A). These force vs. time traces contain a wealth of energetic and kinetic information about the folding process of the protein. Using the Boltzmann relation, differences in free energy between the stable states I_1 , I_2 , and U can be calculated from the population probability histograms (Fig. 2B, see Table 1 and Eq. S7). Further information about the equilibrium free energies can be obtained by exploiting the Crooks fluctuation theorem (17, 18) (Fig. 2C). The intersection (Red Circle) of folding (Blue) and unfolding (Black) work distributions obtained from nonequilibrium force vs. trap separation curves (Inset) defines the equilibrium free energy

of folding of the complete LZ26 coiled coil of $(75 \pm 3) k_B T$ (see Table 1, Eq. S8, and Fig. S2).

The distributions of dwell time intervals τ (Fig. 2D, Inset) allow extraction of rate constants for transitions between I_1 , I_2 , and U. For extracting zero force rates it is important that movements of the transition state under load are modeled correctly. Such transition state movements were neglected in the simple Bell-type model (19), however several improved models have recently been proposed to extract transition state positions as well as zero force folding and unfolding rates from force dependent rate measurements (Fig. 2D) (20–23). We adapted a model initially proposed to describe folding under load (22) to model both folding and unfolding rate constants (*SI Text*). In brief, the unfolded confor-

Table 1. Energetic and kinetic parameters of the LZ26 coiled coil.

| Transition | Dwell time | | | | | Contour length | Probability distribution |
|---------------|-------------------------------|---------------------------------|--|-----------------------------|-----------------------------|------------------------------|--|
| | k_u^0 (s ⁻¹)* | k_f^0 (s ⁻¹)* | ΔG^0 (k _B T) [†] | Δx_u^\ddagger (nm)* | Δx_f^\ddagger (nm)* | Δx (nm) [‡] | ΔG^0 (k _B T) [‡] |
| N, I_1 | – | – | – | – | – | 9.3 ± 1.1 | 9.6 ± 1.1 |
| I_1 , I_2 | $(8.7 \pm 8.3 - 6.9) 10^{-4}$ | $(6.9 \pm 4.1) 10^7$ | 25.1 ± 2.0 | 8.7 ± 0.7 | 8.3 ± 0.1 | 19.9 ± 1.3 | 23.8 ± 0.4 |
| I_2 , U | $(2.5 \pm 1.8) 10^{-5}$ | $(1.7 \pm 4.3 - 1.2) 10^{12.5}$ | 38.8 ± 2.5 | 9.8 ± 0.4 | $24.9 \pm 1.7^{\S}$ | 33.2 ± 1.3 | 42.1 ± 0.4 |
| | – | $(5.0 \pm 2.8) 10^{3.1}$ | – | – | $7.9 \pm 0.6^{\S}$ | – | – |
| N, U | – | – | – | – | – | 62.4 ± 1.0 | $75 \pm 3^{\parallel}$ |

*Errors are a combination of statistical and systematic errors due to trap stiffness uncertainty.

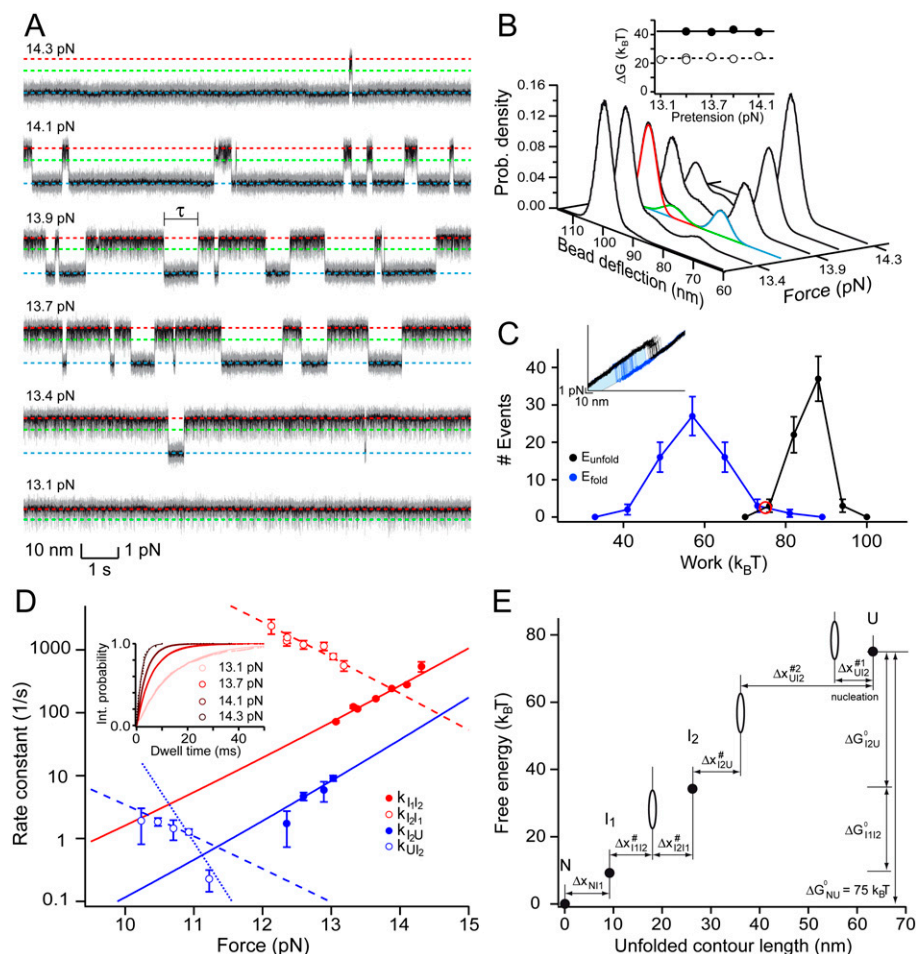
[†]From the ratio of zero force rate constants.

[‡]Errors are \pm SD.

[§]Fit to rate constants above 10.9 pN.

[¶]Fit to rate constants below 10.9 pN.

^{||}From Crooks' fluctuation theorem.



This lower bound is slightly higher than lower boundaries for the preexponential factor ($(2.7\text{--}5.5) \times 10^3 \text{ s}^{-1}$) calculated from measurements of the upper limit of the transition path time (33, 34).

The open and closed symbols in Fig. 3C correspond to the schematic energy landscape of Fig. 2E using our measured preexponential factor for the barrier height estimates. The directly measured landscape and the key check-points of the schematic landscape are in very good agreement. Notably, all barrier positions are reproduced surprisingly well in the deconvolved energy landscape.

It has been a long-standing debate whether broad barriers observed in protein folding experiments indicate a number of energetically equivalent conformations or rather two or more narrow sequential barriers that cannot be resolved due to resolution issues (35, 36). For the apparently broad major barrier between I_2 and U, located at 40–60 nm in Fig. 3C, the deconvolved energy landscape helps to distinguish between those two scenarios. The two maxima of this barrier with a depression at *ca.* 55 nm strongly argue for the sequential barrier model and justify our earlier analysis with a second transition barrier (two slopes for the open blue symbols in Fig. 2D). Furthermore, it is intriguing that the high energy minimum lies exactly at the position of the weak asparagine residue in the C-terminal zipper domain, in accordance with the positions of the intermediate states I_1 and I_2 . However, it is important to point out that errors of the regions with high energy will be necessarily large due to limited thermal sampling of those regions. This error in part explains the smaller measured barrier heights of the last two barriers compared to the heights calculated from the kinetic parameters. Moreover, because barrier heights seem to be influenced by bead kinetics, the decreasing characteristic frequency of the trapped beads with decreasing force yields systematically decreasing barrier heights with increasing protein extension.

Between states N and I_1 the energy landscape appears linear. Because we do not observe distinct transition events for the N to I_1 transition, a barrier, if it exists, has to be lower than the barrier between I_1 and I_2 . The linear shape suggests that I_1 does not represent a real intermediate at higher forces, because the thermodynamic state N represented by a local minimum in the energy landscape vanishes under load. An alternative interpretation would view I_1 as the new native state under load.

Back transformation of the folding free energy landscape to zero force conditions provides the energy landscape shown in Fig. 3D, exhibiting a remarkably detailed picture of the LZ26 leucine zipper stability and folding. The free energy of folding of the coiled coil extracted from this zero force energy landscape (approximately $75 k_B T$) corresponds well to the energy derived from the fluctuation theorem of Crooks. The transformed landscape clearly shows a repetitive energy pattern reflecting the composition of LZ26 from three individual GCN4-p1q coiled coils.

Hydrogen exchange measurements have shown that folding of a C-terminally cross-linked GCN4 zipper domain is barrier-limited and thus exhibits two-state behavior (9). The C-terminal GCN4 zipper domain (*Blue*) of our LZ26 construct, which has a free energy of $21 k_B T$, comparable to bulk measurements (9), is in accordance with this finding, albeit with a very low folding barrier. This domain contains the energy contributions of initial seed formation. In contrast to the C-terminal domain, we find the 2 N-terminal domains (*Green* and *Red*) do not exhibit a barrier. Apparently, folding of these domains proceeds in a downhill fashion. It is important to note that the intermediates I_1 and I_2 only exist and are populated in a force experiment where the elastic linkers under load shape the energy landscape in such a way that pronounced minima appear (Fig. 3C and D). It is this unique property of force experiments that allows us to look into the downhill part of a folding protein. We can only conclude about the downhill nature of the pathway populated under load, not

necessarily about the solution pathway. However, as pointed out above, there are arguments why solution and mechanical folding pathways may be similar for a coiled coil. Comparison of the free energies of the downhill folding domains to the free energy of the domain including the seed now allows an estimate of the entropic costs of seed formation of approximately $6 k_B T$. Moreover, the observation of barrierless folding of concatenated leucine zipper coiled coils shows that those domains can be fused seamlessly without energetic prices. This is in stark contrast to the neck coiled coil of kinesin, where a noncanonical N-terminal hydrophobic collar region prevents seamless elongation of the coiled coil (37).

In the energy landscape of Fig. 3D, both N-terminal zipper domains appear clearly separated into two differently stable regions, a stable C-terminal region comprising two heptad repeats and two significantly less stable N-terminal heptads (indicated with light and dark colors in Fig. 3D). The approximately linear energy increases within these regions indicate that neighboring core residues exhibit similar energy contributions. Even though the GCN4 system is one of the best investigated model systems for protein folding, direct evidence for such an energetic asymmetry has so far been missing. Interestingly, the point of division into the differently stable regions coincides with the location of the asparagine residue in the center of each GCN4-p1q domain. This asparagine residue has been found to be destabilizing for the total free energy of the leucine zipper (16). Whereas this colocalization may be coincidental, a noncanonical residue at the border between two regions of different stability is remarkable. Our results of an energetic asymmetry between the N- and C-terminal heptads can explain experiments that found that C-terminal heptads are more sensitive to mutations of core residues than the N-terminal heptads (38) on an energetic basis. The energetic asymmetry also offers an energetic explanation for the proposed C-terminal trigger sequences governing folding of many physiologically important coiled coil structures (39, 40).

Recently, Li and coworkers proposed a mechanism for protein folding against force in which the polypeptide chain folds via a cooperative collapse of the complete polypeptide chain (41). Using force-clamp AFM they observed slow (0.01–10 s) and continuous folding transients from the unfolded to the folded state. In contrast, even under mechanical loads, the transition times we measure occur extremely fast (approximately 10^{-4} s). Such rapid transitions have also been observed recently in single molecule fluorescence experiments of folding proteins (34). The observation of a large transition barrier far from the native state at large protein extension precludes the formation of a collapsed state at the onset of GCN4 folding.

In this study we demonstrated that single molecule deconvolution equilibrium sampling can reproduce the full distance-resolved energy landscape of a protein and reveal energy modulations even on the native side of the folding barrier. We anticipate that this method will find numerous applications to measuring energy landscapes of proteins to yet unprecedented detail.

Methods

Experimental Procedures. The coiled coil construct is derived from the GCN4-p1 leucine zipper domain (8, 11, 14): MASR MCLEEQK VEELLQK NYHLEQE VARLKQL VGELEQK VEELLQK NYHLEQE VARLKQL VGELEQK VEELLQK NYHLEQE VARLKQL VGECEGL (construct LZ26). The coiled coil was cross-linked via C-terminal cysteines. A second N-terminal cysteine pair at position B of the heptad repeat was used for handle attachment as introduced by Cecconi et al. (3) (see *SI Text* for details). Antidigoxigenin beads sparsely covered with protein DNA constructs and Neutravidin beads (both 1 μm , distinguished by fluorescence) were trapped and brought into close proximity to build a bead-DNA-protein dumbbell. Experiments were performed in PBS buffer in a custom build dual beam optical tweezers setup (see *SI Text* for more information).

Data Analysis. Analysis was done on the difference signal of both beads to increase the signal to noise ratio (42). The force is not constant in our measurements. Every length change of the protein will be associated with a change in tension. A correction for the change in forces is therefore included in all calculations (see *SI Text* for more information).

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