Single Molecule Unzipping of Coiled Coils: Sequence Resolved Stability Profiles

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We use a high resolution atomic force microscopy technique to mechanically unzip and rezip single coiled-coil proteins. This allows us to read off the complete stability profile of the protein turn by turn. We investigated three coiled coils with different length as well as a point mutation and find force fluctuations between 9 and 15 pN that can be directly related to the amino-acid sequences. An equilibrium model previously applied to DNA fully describes the mechanical unzipping process including free-energy contributions of the individual turns and seed formation energy.

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Proteins are polypeptides that fold by a self-assembly process into complex functional molecular machines. This self-assembly process is still far from being understood. Since force is an ideal control parameter to explore the multidimensional energy landscapes of proteins, single-molecule mechanical experiments have become an important tool for studying protein folding [1]. One of the advantages in a force-induced protein-unfolding experiment is that unfolding occurs along a well-defined reaction coordinate, the direction of pulling. By mechanically loading an individual protein domain along a specific direction, properties of the energy landscape projected on the reaction coordinate can be explored [2,3]. The complexity of protein folding has made it necessary to find simple, yet physiologically important model systems to study the process. One of the most important simple model system widely investigated in bulk assays is the coiled-coil structure [4–8]. Coiled coils consist of two polypeptide strands that each form an α-helix. The two α-helices wrap around each other and form a superhelix. Coiled coils possess a well-defined repetitive seven amino-acid sequence motif called heptad repeat $(abcdefg)_n$. Positions a and d in this sequence are mainly hydrophobic residues which form the interface for the dimerization of the two α-helical strands. In the present study we designed a mechanical unzipping experiment that allows us to read off the complete stability profile along the coiled-coil sequence. We show that we can achieve a spatial resolution of four α-helix turns (two heptads) with an energy sensitivity of one k_BT. The design of our experiment is shown in Fig. 1(a). We fused the coiled-coil sequence to the c terminus of five globular protein domains from the actin cross-linker *dictyostelium discoideum filamin* (ddFLN1-5). The coiled-coil sequence leads to dimerization of ddFLN1-5 to form the construct schematically shown in Fig. 1(a). In this experimental geometry ddFLN1-5 serve as handles to anchor the coiled coil between tip and substrate in the desired unzipping geometry. In addition, it provides a characteristic fingerprint in force-extension traces that allow identification of true single-molecule events. Mechanics of ddFLN1-5 have been extensively investigated before [9]. Domain 4 in the ddFLN1-5 molecule unfolds via a mechanical unfolding intermediate, characterized by a double peak in the force-extension traces. We exploit this special unfolding behavior of the domain ddFLN4 to identify dimerized molecules

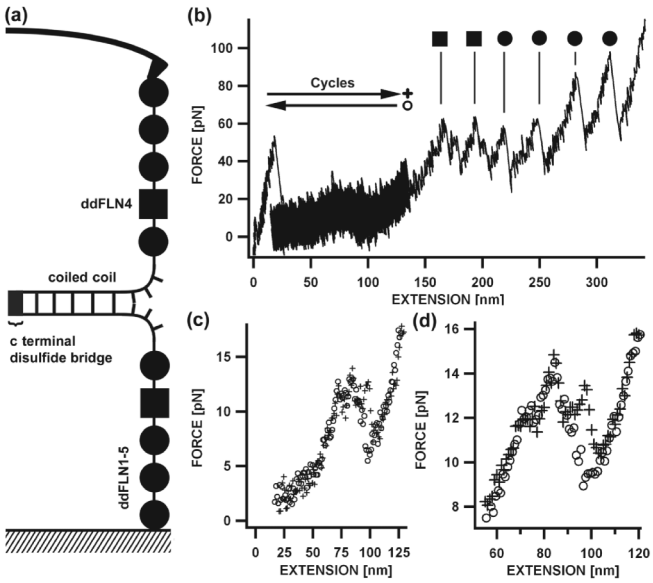

FIG. 1. (a) Schematics of the experimental single-molecule setup for unzipping a double-stranded coiled coil. Binding of the ddFLN1-5 domains (beads) to substrate and tip is unspecific and does not necessarily occur at ddFLN1. DdFLN4 is shown as a black square. The ladder illustrates the coiled-coil construct. (b) Representative force-extension trace of the ddFLN1-5-LZ26 construct. The sawtooth pattern arises from ddFLN1-5 domain unfolding. The unfolding events of ddFLN4 are marked with squares. In each force trace the molecule was stretched and relaxed consecutively up to 14 times in an extension range between 20 and 140 nm since this is the expected range for coiled-coil unzipping. (c) To increase signal-to-noise ratio those stretching traces (crosses) and relaxation traces (circles) were averaged separately. Around 12 pN specific force fluctuations are visible reflecting the coiled coil unzipping and rezipping behavior. (d) A zoom into the force fluctuation pattern with further increased signal-to-noise ratio obtained by averaging five traces from different molecules.

which are anchored in the desired unzipping geometry by discarding all recordings which do not show two unfolding patterns of ddFLN4. In our experiments we investigated three different coiled-coil sequences based on the leucine zipper from the yeast transcriptional activator GCN4-p1 [10]. In addition to the single leucine zipper containing 10 α-helix turns we also investigated double and triple length zippers by repeating the sequence of the single zipper. The three final zippers contained 10, 18, and 26 α-helical turns, respectively. In the following we will refer to the three sequences as LZ10, LZ18, and LZ26 [11]. A typical force-extension trace obtained with the ddFLN1-5-LZ26 construct is shown in Fig. 1(b). At extensions below 150 nm the unzipping of the LZ26 coiled coil is almost masked by the thermal noise amplitude of the force probe. To increase experimental sensitivity we consecutively unzipped and rezipped the leucine zipper up to 14 times within one force-extension trace. To avoid complete α-helical strand separation under force we cross-linked the c-terminal end of the leucine zipper by a disulfide bridge introduced via cysteines. Following the unzipping and rezipping cycles, further extension of the molecule beyond 150 nm now leads to unfolding of the ddFLN1-5 handles resulting in a characteristic sawtooth pattern. Marked with squares are the unfolding events of the two domains ddFLN4 in the dimerized construct. To increase the signal to noise ratio in the unzipping region we now averaged the unzipping and rezipping traces separately. Such an averaged force-extension trace is shown in Fig. 1(c). It is important to note that this averaging procedure is only possible with systems very close to equilibrium. Since the averages of unzipping as well as rezipping traces are almost identical the averaging procedure is justified. Further noise improvement was then achieved by averaging five such experimental pre-averaged traces [see Fig. 1(d)].

Force-extension traces of all three leucine zipper constructs obtained by this procedure are shown in Fig. 2. Each trace reveals a specific unzipping pattern with fluctuating force between 9 and 15 pN. Applying force to the zipper in our experimental geometry allows to populate states with increasing degree of unzipping within the leucine zipper energy landscape. Those partially unzipped conformations are almost inaccessible in conventional assays due to the two-state folding behavior of the mechanically unconstrained protein [5,12,13]. Can we relate the measured equilibrium unzipping force-profile to the leucine zipper sequence? To this end we adapted an equilibrium model previously described for DNA unzipping experiments by Bockelmann *et al.* [14]. In brief, we calculated a simplified partition function of the system containing cantilever, elastic polymer spacer, and partially unzipped leucine zipper. The thermal average of an observable, e.g. force, can be written as follows:

$$\langle F(x_0)\rangle = \frac{\sum_{j,z} F(j, z, x_0) e^{-E_{\mathrm{tot}}(j,z,x_0)/k_B T}}{\sum_{j,z} e^{-E_{\mathrm{tot}}(j,z,x_0)/k_B T}}, \qquad (1)$$

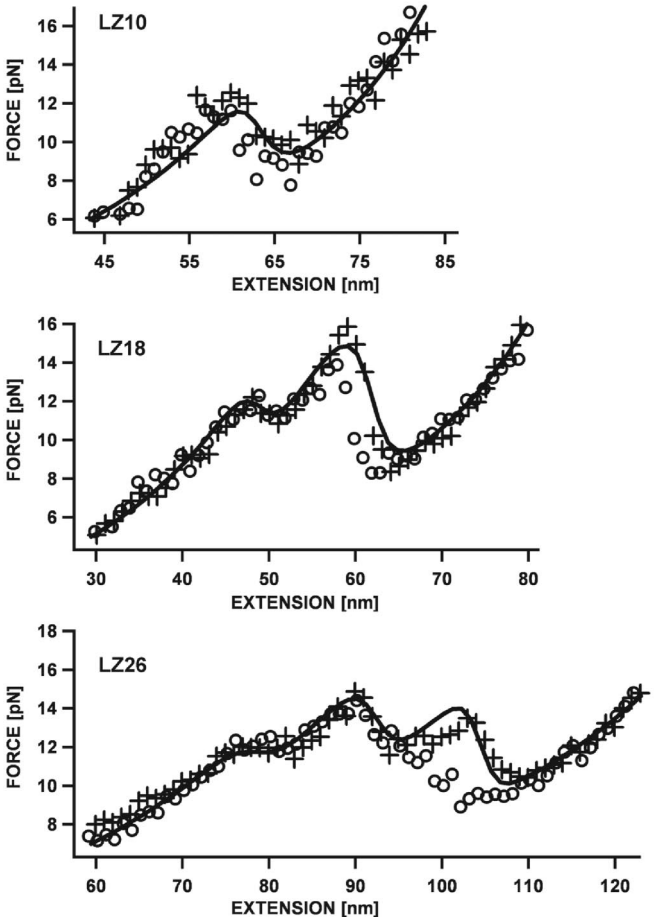

FIG. 2. Unfolding (crosses) and folding (circles) force-extension traces of the three investigated coiled coils compared to the theory. The mean pulling velocities are 460, 510, and 690 nm/s for the LZ10, LZ18, and LZ26 coiled coils, respectively.

where x_0 is the distance between substrate and equilibrium position of the unloaded cantilever. For each fixed x_0 the summation ranges over all possible cantilever tip positions z and all possible unzipped conformations of the leucine zipper j. The energy for each state is given by $E_{\mathrm{tot}}(x_0, z, j) = E_{\mathrm{unz}} + E_{\mathrm{ext}} + E_{\mathrm{lev}}$. E_{ext} is calculated from the interpolation formula of the wormlike chain model for polymer elasticity [15] with a persistence length of $A = 0.7$ nm. For simplicity we classified the leucine zipper sequence into units of single α-helical turns (3.5 amino acids). The contour length gain per unfolded turn is 2.59 nm. E_{unz} is the energy needed to unzip j turns, where the only fit parameters are the sequence depending energies for opening each single turn E_{turn}. Table I gives the mean values for E_{turn} gained from a Levenberg-Marquardt fit to our data, which converged robustly for a broad range of start parameters. We named the energies associated with opening a turn by the amino acid located in the nearest a or d position of the sequence [underlined in [11]]. The leucine zipper sequence then exhibits four different classes of

TABLE I. Energies E_{turn} for the unzipping of a single α-helical turn obtained from fitting Eq. (1) to the data (see Fig. 2). The turns are named by the amino acid in the hydrophobic core of the coiled coil at position a or d within the turn [underlined in [11]]. The ΔG values shown were taken from [16]. Zero energy levels for the ΔG values are arbitrary and were chosen so that Leucine/Valine values of E_{turn} match the ΔG value.

Turns	Leu (L), Val (V)	Met (M)	Asn (N)	Nucleation seed (C)
E_{turn} (k_BT)	4.42 ± 0.2	1.9 ± 1.1	-1.5 ± 0.9	-7.4 ± 1.4
ΔG (k_BT)	4.4	3.2	-1.1[a]	

[a]From amino-acid exchange experiments on GCN4, the asparagine value is $-1.6k_BT$ [17].

turns: turns containing either Leucine (L) or Valine (V), turns containing Asparagine (N), a turn containing Methionine (M), and a turn containing the c-terminal cysteine (C) whose E_{turn} value includes nucleation seed energy. The model completely reproduces our data obtained for all three different leucine zipper sequences with one fixed set of energy contributions E_{turn} shown in Table I. The energies we find are in close agreement to ensemble studies with point mutated leucine zippers [16–18] (see Table I). Additionally, the total stability for LZ10 of $24k_BT$ from our fit agrees well with a value of $20k_BT$ from bulk studies with an LZ10 zipper shortened by three amino-acid residues [19]. It is interesting to note that single-molecule unzipping allows to directly read off the spatially resolved stability profiles without the necessity of mutational analysis. At first sight, a comparison between ΔG values obtained in ensemble studies and E_{turn} values used in the partition function [Eq. (1)] does not seem straightforward. However, the values E_{turn} already contain many important molecular entropic contributions like hydration or flexibility of side chains since the simple partition function only accounts for the trivial entropic contributions from polymer elasticity. The E_{turn} values are hence likely very similar to ΔG values.

The force profiles of all three leucine zippers show pronounced minima and maxima. The equilibrium model allows to identify the minima with turns containing the hydrophilic asparagine in an a position of the zipper. This reflects the important contribution of amino acids within the hydrophobic core to the overall stability of the zipper. The model also allows to predict the influence of point mutations on the mechanical stability profile. We tested this result by mutating the asparagine within turn five to valine in LZ18. This mutation eliminates the corresponding dip in the force-extension trace and leads to the expected stability increase of $5.9k_BT$ (see Fig. 3).

While the experimental sensitivity allows detecting effects of point mutations down to one k_BT the spatial resolution of our method depends on the spring constants of both force probe and the tethered polypeptide. The weakest spring (force probe or polypeptide) will dampen propagation of force fluctuations and hence limit resolution [20,21]. The leucine zipper inherently contains a polypeptide spacer that grows in length with increasing degree of unzipping which will ultimately limit resolution. For the experiment we chose the cantilever stiffness (6–8 pN/nm) such that it lies above the polypeptide spacer stiffness at the typical unzipping forces (0.5 pN/nm). Hence resolution is mainly limited by the soft polypeptide spacer. We determined spatial resolution in analogy to the Rayleigh criterion in optics as the closest distance of two stability fluctuations within the sequence that we can resolve mechanically. Using a model sequence of two sharp free-energy dips with variable distance from each other we could estimate a minimal resolvable distance of 4 turns (14 amino-acid residues) in the sequence. Therefore the width of the characteristic force dip at the asparagine positions for LZ18 and LZ26 is dominated by the resolution limits of the unzipping experiment. The true cooperative length of stability fluctuations within the sequence would only be accessible in a hypothetical experiment using an infinitely stiff force probe and infinitely stiff polypeptide spacer.

The c-terminal force drop leading to complete unzipping for all three leucine zipper constructs (Fig. 2) is much more pronounced than expected for a merely weak asparagine containing turn. In addition to the weak asparagine turn this

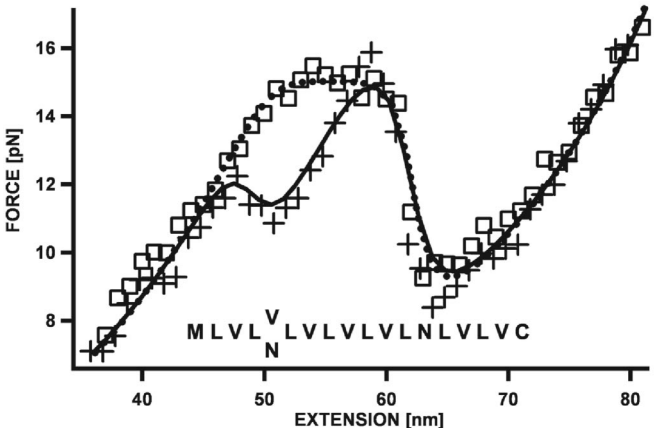

FIG. 3. Calculated force-extension trace of the LZ18 coiled coil (solid line) compared to the data (crosses) as in Fig. 2. Overlaid is the calculated force-extension trace of a LZ18 mutant where the asparagine in turn five was replaced by a valine (dotted line) and the experimental data (quadrates). The difference between the force-extension traces from both zippers are localized at the n terminus while the c-terminal force-extension behavior is not affected.

reflects also the unfavorable energetics of seed formation occurring at the c-terminal end of the leucine zipper [6,8,19]. The fits from the equilibrium model provide a direct measure for the free-energy cost of this seed formation of $-7.4k_BT$. This allows the conclusion that the minimal size for a leucine zipper construct with positive energy is at least three turns ($-7.4k_BT$ for the seed $+2$ times $4.4k_BT$ for regular stabilizing turns). If such a construct contained a weak asparagine turn the minimum size would even grow to five turns (Table I). This estimate is in good agreement with bulk studies on non-cross-linked GCN4-p1 subdomain leucine zippers [22]. So far, we have discussed our results entirely in the framework of an equilibrium model. As pointed out, equilibrium is a good assumption, since stretch and relax traces are virtually indistinguishable at almost all extensions. However, there is a noticeable hysteresis at large extensions where the coiled coil is completely unzipped in the stretch traces and tries to nucleate in the relax traces. The LZ26 construct exhibits a more pronounced hysteresis than the shorter constructs (see Fig. 2). A possible explanation for these results lies in the higher pulling velocities at which the longer construct was investigated. A systematic study of nucleation kinetics will be an important task for the future.

In conclusion, we could show that mechanical equilibrium unzipping of a protein coiled-coil structure allows to read complete sequence-resolved stability profiles in one experimental run. Since coiled coils are important dimerization motifs in many cellular proteins we anticipate that the method presented here will contribute significantly in understanding these simple and yet far from predictable protein structures.

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