Protein Folding

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Surprising Simplicity in the Single-Molecule Folding Mechanics of Proteins**

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Over the last ten years, single-molecule force spectroscopy has proven to be extremely useful in studying the unfolding-energy landscapes of proteins.[1,2] One major advantage of this new approach is the precise control of the reaction coordinate. In earlier force spectroscopy experiments, the reaction coordinate was mainly constrained to the N–C-terminal direction of the protein. However, recently the toolkit to design pulling geometries along almost arbitrary force directions was extended by disulfide engineering of polyproteins.[3] In those experiments, a strong anisotropy of the unfolding-energy landscape was observed. Unfolding rates varying by several orders of magnitude were found along the various pulling directions.[4] To date, the effects of force on the folding pathway have only been rarely studied, owing to the much lower forces involved in active refolding and the associated technical demands.[5–7] Herein, we describe the design of single-molecule experiments to study the anisotropy of the folding mechanics of a protein under external force.

The idea and experimental realization of our experiment is depicted in Figure 1. The conventional geometry for studying the mechanics of protein folding is shown in the scenario at the top (blue). A polypeptide chain is held at its N and C termini, and hence the mechanical force will act on the whole chain while the protein is folding. To study the effect of force on protein folding, it would be desirable to compare the N–C-terminal pulling geometry with other geometries in which the mechanical force only acts on part of the chain (middle and bottom scenarios in Figure 1). We used the protein ubiquitin, which has been characterized in unfolding and refolding experiments.[6,8] Recently, it was shown that ubiquitin folds against mechanical loads applied in the N–C-terminal direction. To realize the three pulling geometries of Figure 1, we used cysteine engineering, which allowed us to change the sites of force application. The force was applied through residues 1 and 76 in the first pulling geometry (blue), 1 and 35 in the second geometry (red), and 1 and 16 in the third geometry (green). The parts of the polypeptide chains exposed to force during folding are colored in the three

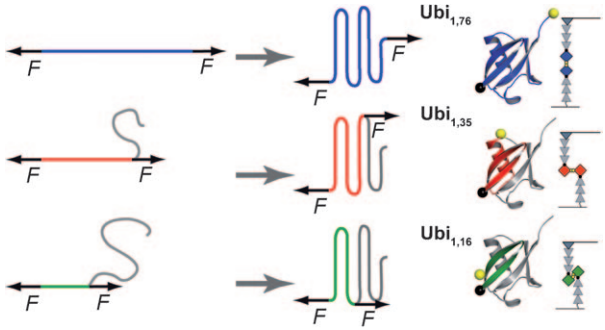

Figure 1. Anisotropy of folding mechanics under force. The conventional design of force experiments between the N and C termini is illustrated in the top scenario (blue). Different pulling directions result in a partly constrained polypeptide chain during active folding and are shown in the middle and bottom scenarios (red and green). The protein ubiquitin allowed the experimental realization with the three shown constructs. The attachment to the surface and the cantilever was achieved through Ig-handles (gray triangles).

protein structures shown in Figure 1. Attachment of the N terminus of ubiquitin to the cantilever tip and the surface occurred through three immunoglobulin (Ig) domains of human titin (I91–I93) fused to the N terminus of ubiquitin. On average, the titin domains unfold at higher forces, while refolding occurs with kinetics one to two orders of magnitude slower than for ubiquitin.[9] The cysteine residues introduced into ubiquitin at positions 76, 35, or 16 ensured dimerization, resulting in constructs as shown in the rightmost column of Figure 1. The unfolding fingerprint of the two ubiquitin domains sandwiched between Ig domains was clearly observable in unfolding traces (see the Supporting Information).

We used the following protocol for mechanical refolding experiments: First, both ubiquitin domains and one to three Ig-handle domains were unfolded. Afterwards, the unfolded, stretched polypeptide chain was relaxed with a continuous velocity $v_p = 5\ \mathrm{nm\,s^{-1}}$ down to an extension of approximately 20 nm above the surface. Subsequently, the polypeptide chain was stretched again with the same pulling velocity back to the starting extension. To minimize drift artifacts in the force–extension traces and to increase the force resolution, we performed these experiments with a lock-in detection adding a small oscillation amplitude of 7 nm on the tip movement, as described by Schlierf et al.[7] (see also the Supporting Information). This additional lock-in signal can be used in cases for which instrumental drift complicates identification of refolding events. Those folding events can be identified by clear, discrete events in the lock-in traces (see reference [7] and the Supporting Information). Figure 2 a–c shows typical force–extension folding traces (colored) and subsequent unfolding traces (gray) for the three different ubiquitin constructs $Ubi_{1,76}$, $Ubi_{1,35}$, and $Ubi_{1,16}$. All traces exhibit two

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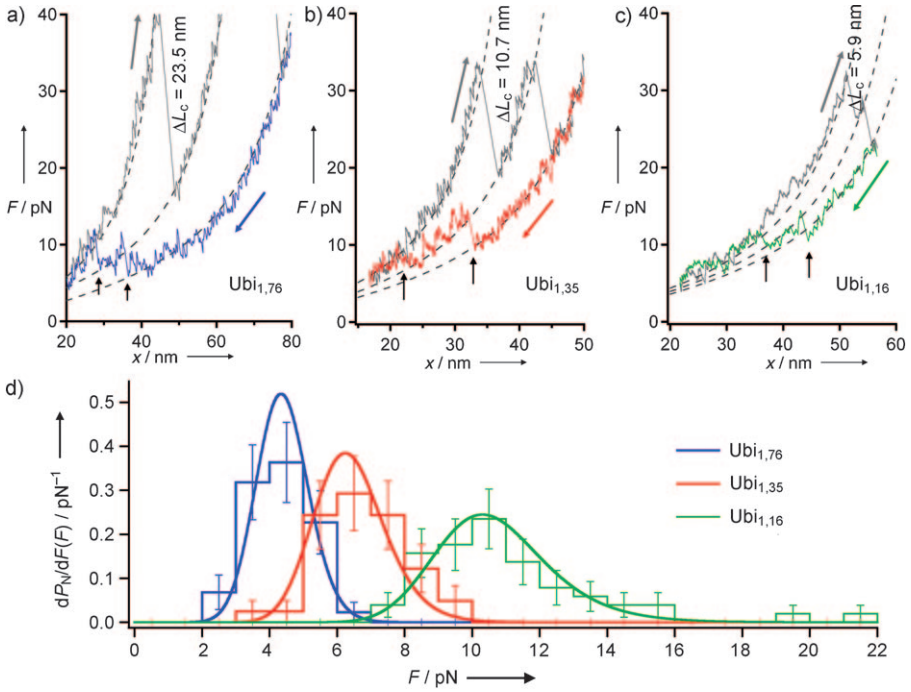

Figure 2. Active protein folding forces in different pulling directions. a) Typical $Ubi_{1,76}$ refolding (blue) and subsequent unfolding (gray) force–extension trace. During the relaxation of the unfolded $Ubi_{1,76}$ dimer, two clear refolding events (black arrows) were detected. b) Typical $Ubi_{1,35}$ refolding (red) and unfolding force–extension trace. The refolding events (black arrows) were detected at higher forces than in (a). c) Typical $Ubi_{1,16}$ refolding (green) and unfolding force–extension trace. Even though the contour length change ΔL_C is the smallest in this construct, the refolding events (black arrows) were easily detected owing to their high average forces. d) Experimental refolding force distributions of $Ubi_{1,76}$ (blue, $n=44$), $Ubi_{1,35}$ (red, $n=41$), and $Ubi_{1,16}$ (green, $n=51$). The corresponding lines are the calculated force distributions based on a simple energetic-barrier model.

distinct refolding peaks (black arrows) at low forces and two subsequent unfolding peaks at higher forces. An analysis of contour length change allows us to attribute the folding and subsequent unfolding events to the individual ubiquitin domains pulled at the three different attachment points. Expected contour length gains for the three attachment geometries are $\Delta L_C = 24$ nm for $Ubi_{1,76}$, $\Delta L_C = 10.9$ nm for $Ubi_{1,35}$, and $\Delta L_C = 5.3$ nm for $Ubi_{1,16}$. We find values of $\Delta L_C =$ 23.5 nm, $\Delta L_C = 10.7$ nm, and $\Delta L_C = 5.9$ nm, respectively.

In earlier folding studies of only the N–C-terminally linked ubiquitin ($Ubi_{1,76}$) using an AFM force clamp, a highly complex folding behavior of ubiquitin under force was reported.[6] Specifically, those authors reported that folding of a polyprotein chain of several ubiquitin units occurred through a cooperative collapse rather than through discrete refolding events of individual domains, as expected for a mere two-state folder.[6] In contrast, we observe discrete folding events for the individual ubiquitin domains. Our findings are in good agreement with results from molecular dynamics simulations.[10]

From the refolding events we compiled the refolding force distributions shown in Figure 2d. We define refolding force as the force at which the refolding event starts, that is, the troughs of the refolding peaks marked by the arrows in Figure 2a–c. $Ubi_{1,76}$ exhibits the lowest refolding forces $F_{avg} = 4.0 \pm 0.9$ pN ($n=44$) as well as the narrowest distribution (blue). The average active folding force of $Ubi_{1,35}$ increases to $F_{avg} = 6.8 \pm 1.2$ pN ($n=41$) with a wider distribution. The shortest construct ($Ubi_{1,16}$) shows the highest average refolding force $F_{avg} = 11.2 \pm 2.7$ pN ($n=51$) as well as the widest force distribution. Apparently, the constructs for which shorter parts of the polypeptide chain are subject to force fold at higher forces than the one with whole-chain attachment. How can this dependence be understood? Simple models of protein folding under load have been put forward recently.[7,11] In those models it is assumed that forces acting on a polypeptide chain increase the unloaded barrier of folding owing to the additional costs of contracting the polypeptide against load. Our data now allow direct testing of those models.

As described in detail in reference [7], we calculated the force-induced energy barrier as a function of the polypeptide spacer length and elasticity and of the cantilever stiffness. The folding force distributions can now be derived without any fitting parameters, as the folding rate in the absence of force ($k_f(F=0) = 200\ s^{-1}$)[12] and the polypeptide elasticity of the unfolded chain[7] are known. We calculated the three expected folding force distributions for $Ubi_{1,76}$, $Ubi_{1,35}$, and $Ubi_{1,16}$ using the various lengths of the loaded parts of the polypeptide chains of the respective constructs. The agreement between the experimental data and the predicted distributions is remarkable (Figure 2d). It is important to note that not only the average refolding forces but also the distribution widths agree almost perfectly. Surprisingly, the folding mechanics of a protein with various attachment points along the polypeptide chain can be described using merely a single force-free folding rate and the entropic polymer elasticity of the unfolded polypeptide chain. In contrast to the unfolding mechanics, refolding forces seem to be independent of the details of the final tertiary structure and of the details of the amino acid sequence as long as they do not change polypeptide elasticity. Refolding forces of proteins can hence be rationally designed by changing the attachment points. Since the simple mechanism we find for load-dependent folding of ubiquitin could also be successfully applied to the folding of ddFilamin,[7] we anticipate that our model may be generally applicable to describe protein folding under load.

Naturally, the simplicity of the folding-energy landscape will only hold for two-state folders. In the case of multistate

folders, analysis of refolding distributions will give insight into the structure and dynamics of such folding intermediates. Recently, we showed that a folding intermediate of the actin crosslinker ddFilamin can increase the active folding force and thus help the protein to acquire its native structure under strained conditions.[7]

In conclusion, we have shown that force affects the refolding kinetics of a protein by introducing an additional potential barrier through the energetic costs of contracting the chain against force. The folding kinetics are well reproduced by a single force-free rate constant and the number of actively contracting amino acids. We anticipate that designing different points of force application along the folding polypeptide chain will provide an important tool for characterizing folding intermediates along the folding pathways of proteins.

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