Protein Folding

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Navigating the Folding Energy Landscape of Green Fluorescent Protein**

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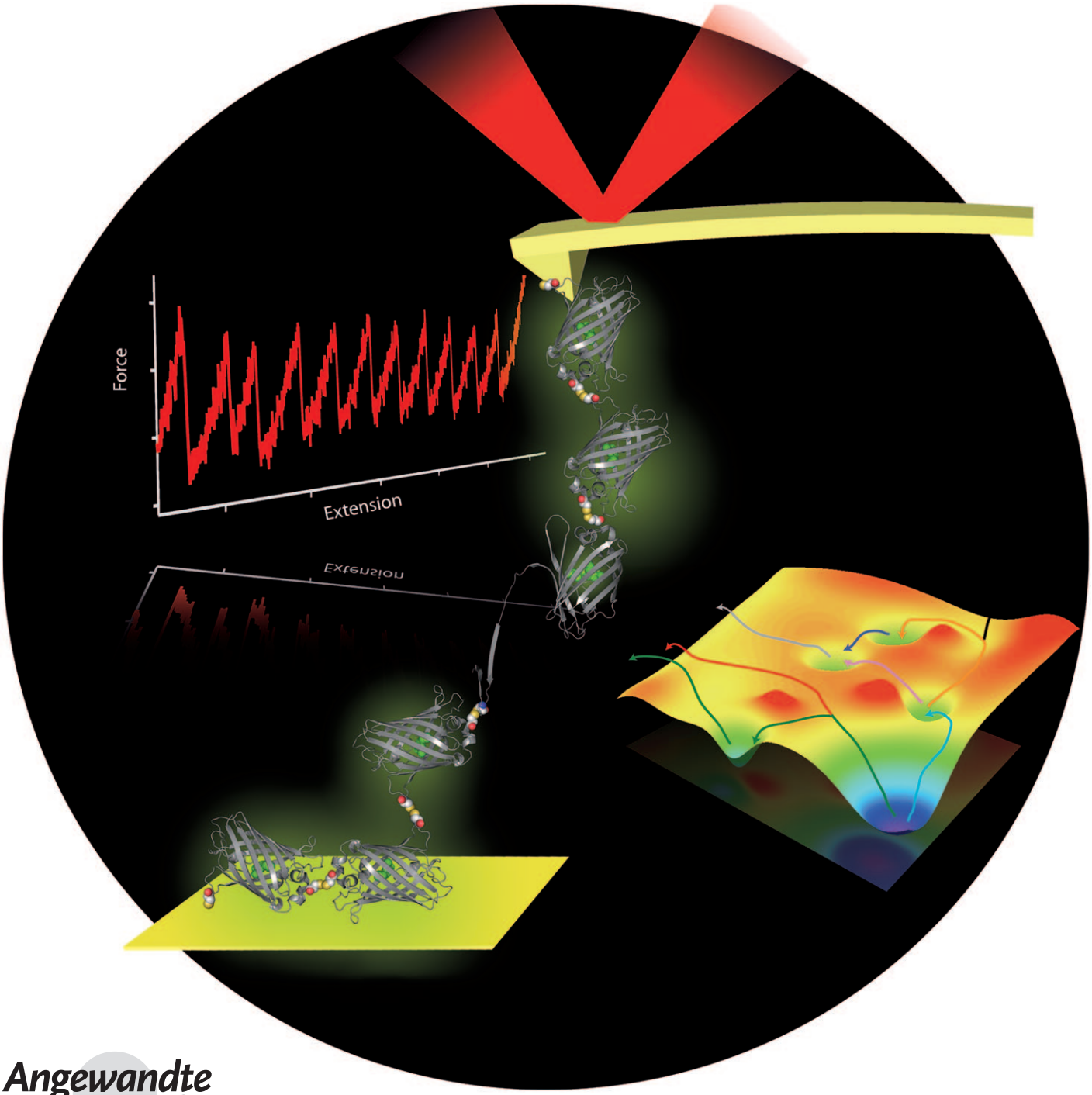

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Protein folding is a complex process in which the polypeptide chain adopts a well-defined structure starting from a large ensemble of more expanded conformations. To describe the folding pathway of a protein in its energy landscape researchers often use denaturants or temperature to induce conformational changes between the folded and unfolded states. However, in a cellular context, conformational changes of a protein are controlled by mechanisms very distinct from chemical and thermal denaturation. Examples include forced unfolding and subsequent import into cellular compartments or into dedicated protein-degradation machines. Hence, it is conceivable that these various folding, refolding, and unfolding schemes are distinct from each other.[1]

As opposed to chemical denaturation, mechanical force acts as a vector on the protein investigated. Thus, single-molecule force spectroscopy can locally probe the energy landscape of a protein, steer the unfolding pathway, and provide direct information about unfolding pathways and populated intermediate states. In addition, structural information about these intermediates is available from contour length gains.[2–6] Mechanical experiments with green fluorescent protein (GFP) have revealed that, when it is pulled at its termini, a small N-terminal α-helix detaches.[2] Two additional intermediate states ensue where N-terminal β-sheets are successively removed. The structures of these intermediates were corroborated by molecular mechanics simulations.[3] Interestingly, also in bulk folding studies an intermediate structure was reported; however, it is structurally very different from the mechanical intermediates.[7,8] A priori, this difference is not surprising since one would expect the pathways to be very different in those two experiments.

Recently, pairwise cysteine mutations on the surface of GFP have been used to construct disulfide-linked polyprotein chains in defined linkage geometries.[9] The position of the cysteine residues defines the points of force application onto the protein's tertiary structure. When GFP is pulled between residues 3 and 212 another mechanical unfolding intermediate is observed,[9] but no structural information about this intermediate is available.

Herein we describe how changing the direction of force application can be used to rationally switch the unfolding of GFP between two distinct pathways with distinct unfolding intermediates. We show that the pathway where GFP is pulled in a shearing geometry resembles a "folding-like" pathway. The other pathway, where GFP is pulled in an unzipping geometry, may rather be relevant for processes resembling cell import.

Previous experiments have shown that when GFP is pulled between residues 3 and 212, a bifurcation in the unfolding pathway is observed:[9] the protein either unfolds in an all-or-nothing manner (red circles, Figure 1 a) or fractures only partially (green circles) with the subsequent unfolding of an intermediate state (blue circles). To obtain structural information about this mechanical unfolding intermediate, we used disulfide cross-links and glycine inserts as local probes. Disulfide cross-links shorten the stretched polypeptide by the number of amino acids bridged by the cysteine residues. Conversely, glycine inserts lengthen the molecule by the

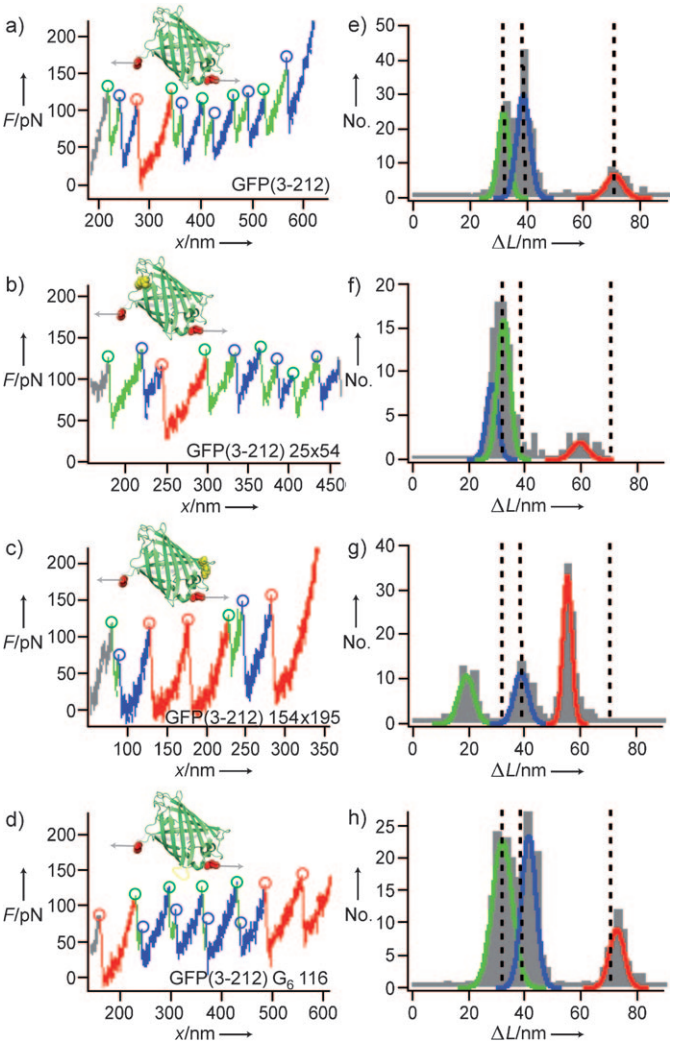

Figure 1. a–d) Typical force–extension traces of GFP(3-212) and GFP(3-212) mutants. Green, blue, and red circles indicate partial GFP fracture, unfolding of the intermediate, and complete fracture, respectively. Subsequent contour length increases are colored accordingly. e–h) Contour length gain (ΔL) distributions for GFP(3-212) and variants (gray bars). Solid colored lines indicate Gaussian fits to the populations (green: partial fracture, blue: fracture of the intermediate state, red: complete fracture). Average values of GFP(3-212) are indicated by broken lines in.

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number of residues inserted. Both probes affect the gain in contour length after unfolding, which can be measured very sensitively.[3,4,6] We constructed two cross-linked variants of GFP(3-212), one with a cross-link in the N-terminal region (GFP(3-212)25 × 54) (Figure 1b) and one towards the C terminus (GFP(3-212)154 × 195) (Figure 1c). Both mutants retain the two-pathway unfolding pattern of GFP(3-212) (see also the Supporting Information). Distributions of the contour length gain of GFP(3-212) and the mutants are shown in Figure 1e–g. Gaussian fits to the three populations are shown as red (complete fracture), green (partial fracture), and blue (fracture of the intermediate) solid lines. If a cross-link is located in the part of the protein that fractures initially, the green population will be shifted to shorter contour length gains. This is what we observe in our mutant GFP(3-212)154 × 195. Conversely, we find that the N-terminal cross-link 25 × 54 shortens the contour length gain upon unfolding of the intermediate state (blue population). Hence, we can conclude that in GFP(3-212) the molecule fractures C-terminally yielding an intermediate with folded residues at the N terminus. The all-or-nothing pathway likely starts from the N terminus (see the Supporting Information).

While cross-link mutations give a coarse picture of the structure of the intermediate, glycine inserts can provide more specific information about single secondary-structure elements. In our mutant GFP(3-212)$G_6$116, we inserted six glycine residues at the N-terminal end of β sheet 6 (Figure 1d). In this mutant, the contour length gain upon unfolding of the intermediate state (blue population) is longer than in GFP(3-212) (Figure 1h). This indicates that β sheet 6 is still folded in the intermediate. We can therefore conclude that the intermediate structure in GFP(3-212) consists of β sheets 1–6 (residues 3-128), consistent with the observed contour length increase of this transition.

A structurally similar intermediate in GFP has been observed recently in equilibrium H/D-exchange experiments[8] as well as during the refolding of acid-denatured GFP.[7] Moreover, the boundaries of our mechanical unfolding intermediate coincide with a split-GFP variant[10] further supporting the importance of this intermediate for folding. Molecular dynamics simulations in combination with experiments have indicated that in isolation this part of the GFP barrel is structured with an already formed chromophore.[11] Recent FRET single-molecule unfolding experiments on the GFP variant citrine have also indicated—albeit without structural information—the presence of two unfolding pathways during chemical denaturation. Here, one pathway proceeds via an intermediate with low fluorescence and one directly to the denatured state;[12] this is strikingly similar to the unfolding behavior we find for GFP in the direction (3-212). It has often been argued that mechanical unfolding pathways are entirely distinct from solution folding pathways. However, our results show that when GFP is pulled at residues 3 and 212, the force-induced unfolding pathway shows remarkable similarity to the equilibrium folding/unfolding pathway.

The unfolding pathway we just described for the direction (3-212) is markedly different from the N-C-terminal pulling direction (3-229) (see Figure 3). In GFP(3-229), first an N-terminal α-helix and then N-terminal β-sheets are sequentially detached from the GFP structure.[2,3] In GFP(3-229) the force acts perpendicular to the β sheets forming the GFP barrel (Figure 3, orange arrow). This allows secondary-structure elements to successively unzip from the GFP barrel. In contrast, in GFP(3-212), the force acts parallel to the direction of the β sheets (Figure 3, green arrow). This leads to the shearing of the β sheets. Evidently, by changing the C-terminal point of force application we steer the molecule into different parts of its energy landscape.

Varying the C-terminal point of force application in such a way that the force acts perpendicular to the β sheets should result in an unfolding pathway very similar to the unzipping-like pathway found in GFP(3-229). To this end, we constructed a mutant where force is applied to residues 3 and 198 (GFP(3-198), Figure 2a). The unfolding behavior observed is

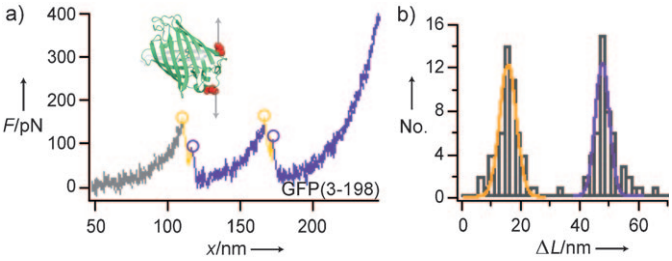

Figure 2. a) Typical force–extension trace of GFP(3-198). The initial unfolding event leading to an intermediate is indicated by an orange circle, the fracture of this intermediate state by a purple circle. b) Contour length gain (Δ*L*) distribution for GFP(3-198). The solid lines indicate a Gaussian fit to the populations (orange: partial fracture, $\Delta L = (15.9 \pm 0.7)$ nm , purple: fracture of the intermediate, $\Delta L = (48.5 \pm 0.7)$ nm).

clearly different from that of GFP(3-212): The molecule fractures partially (orange circles) leading to an intermediate state (purple circles). From this intermediate, the molecule unfolds completely. The total contour length increase for GFP(3-198), (64.6 ± 0.5) nm, is significantly shorter than the expected value of 69.0 nm. A similar length defect was observed in GFP(3-229), and this defect in length was attributed to the unfolding of the N-terminal α-helix at low forces.[2] Additionally, the intermediate we observe in GFP(3-198) is consistent with the detachment of three N-terminal β sheets, an intermediate observed on the unfolding path of GFP(3-229). These two points strongly argue for an unfolding pathway of GFP(3-198) that is very similar to that of GFP(3-229). Whether the unzipping-like pathway has relevance in physiological processes is not yet entirely clear. However, recently, Martin et al. have suggested the possibility that an unzipping-like intermediate may be populated when GFP is pulled through the pore of the AAA+ protease ClpXP.[13]

Figure 3 summarizes the complex energy landscape of GFP that can be sampled in single-molecule mechanical experiments with varying directions of force application. If force is applied perpendicular to the β sheets, the unfolding occurs by means of unzipping. This is the case for GFP(3-229) and GFP(3-198). Both directions share the intermediate

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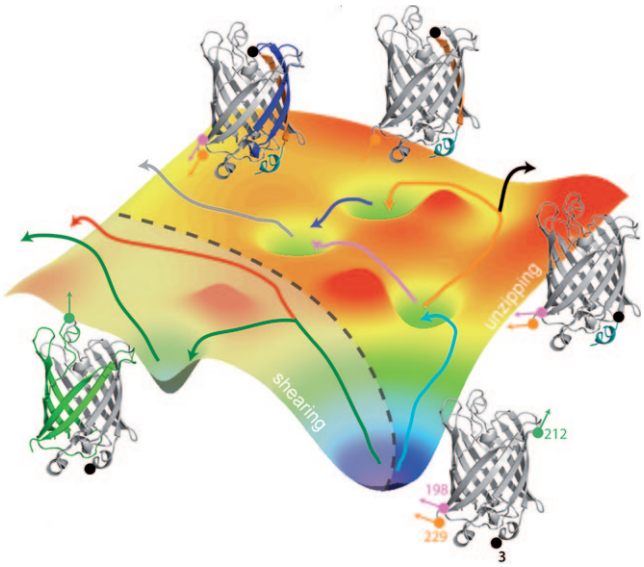

Figure 3. Navigating the complex energy landscape of GFP. While keeping the N-terminal point of force application fixed (black circle), depending on the C-terminal point of force application (orange, purple, and green circles for residues 229, 198, and 212, respectively), GFP can be steered towards different minima in the energy landscape. Detached elements are colored according to the respective transition (colored arrows in the energy landscape), while the structured parts of the intermediates are shown in gray. The shearing region is shaded.

having one α helix (light blue pathway) detached. In GFP(3-229), unfolding then proceeds through two further intermediate states consisting of the GFP barrel with one (orange) and three (dark blue) N-terminal β-sheets detached.[2,3] Alternatively, unfolding from the C terminus leads to the complete breakdown of the structure (black). This bifurcation is bypassed in GFP(3-198) (purple), and the three N-terminal β-sheets are detached simultaneously.

Probing the molecule in a shearing geometry explores an entirely different part of the energy landscape (shaded in Figure 3): C-terminal fracture results in an intermediate state with five C-terminal β-sheets detached (green pathway). In the second pathway, however, fracture occurs in an entirely two-state manner (red). This region of the energy landscape includes an energy minimum populated by GFP under equilibrium conditions in solution[8] and on the folding pathway.[7] Hence, the shearing pathway appears to be a folding-like pathway.

In summary, this study demonstrates the unique possibilities of mechanical forces to explore a protein's energy landscape in an exceptionally controlled way. Our experiments open the way for a broader understanding of protein-folding energy landscapes. In the future, similar experiments can be designed mimicking directly the folding/unfolding processes a protein may undergo during its life cycle in a cell, for example the unfolding of a protein upon transport through pores.[14]

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