Protein Folding

DOI: 10.1002/ange.201405566

Misfolding of Luciferase at the Single-Molecule Level**

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Abstract: *The folding of complex proteins can be dramatically affected by misfolding transitions. Directly observing misfolding and distinguishing it from aggregation is challenging. Experiments with optical tweezers revealed transitions between the folded states of a single protein in the absence of mechanical tension. Nonfolded chains of the multidomain protein luciferase folded within seconds to different partially folded states, one of which was stable over several minutes and was more resistant to forced unfolding than other partially folded states. Luciferase monomers can thus adopt a stable misfolded state and can do so without interacting with aggregation partners. This result supports the notion that luciferase misfolding is the cause of the low refolding yields and aggregation observed with this protein. This approach could be used to study misfolding transitions in other large proteins, as well as the factors that affect misfolding.*

The conformational search of a polypeptide on the way to its native state is a complex process that can involve multiple transitions between partially folded structures.[1] Such intermediate states are more often observed in large proteins of over 100 residues and may progressively guide the polypeptide chain to the native structure. Intermediates may also constitute misfolded states that trap the protein chain, a process that can increase the risk of aggregation.[2] Misfolding and aggregation is associated with diverse medical conditions.[3] It has long been speculated that during folding in vivo, molecular chaperones may suppress entry to misfolded states,[4] rescue proteins that have been already trapped in misfolded states,[5] or target the misfolded proteins for degradation.[6]

Misfolded states have been reported for a number of model proteins.[5,7] At the structural level, misfolded proteins have been studied by using techniques such as Chevron analysis, NMR spectroscopy, Φ-value analysis,[7c,8] and protein engineering combined with computational modeling.[9] Detecting misfolded states during folding is challenging, however, owing to the the highly dynamic and heterogeneous nature of the folding process. Moreover, reversible aggregation can result in kinetics resembling those of folding intermediates or misfolded states. Hence, in folding experiments and in vivo, the formation, stability, and importance of misfolded monomers and their dependence on interactions with aggregation partners often remains unclear. The mechanical manipulation of proteins by optical tweezers is a promising approach since aggregation is impossible when probing a single molecule and transient intermediate folded states can be detected.[7d,10] Off-pathway states could be observed by using this method, although this requires that the protein chains be held under tension to enable length determination and to stabilize the intermediate states.[10–11] The aggregation of two or more monomers has been measured by using protein constructs consisting of multiple head-to-tail monomer repeats.[7e,12]

In this work, we aimed to probe the transitions to a misfolded state and stability of this state in the absence of applied mechanical tension. Mechanical tension is generally also lacking in vivo, as well as in bulk in vitro refolding assays, and can affect the protein conformational search. We interrogated a comparatively large multidomain protein since these features are thought to increase the tendency to misfold and the stability of the folding intermediates,[13] and hence to facilitate detection. The protein luciferase (61 kDa, 550 residues; Figure 1a) has been reported to adopt non-native states upon repeated freeze–thaw cycles[5,14] and has a general propensity to aggregate.[15] Luciferase folding is of general relevance because it is a model substrate for diverse studies on protein folding and chaperone activity.[5,6b,16] To interrogate folding and misfolding transitions in the absence of mechanical tension, we first unfolded individual luciferase proteins with optical tweezers, relaxed them for a specified time to zero tension to allow refolding or misfolding, and finally assessed their new state through stretching.[17] This approach provided information on the transitions between folded states and their lifetimes in the absence of applied force, which in turn can provide information on misfolded states that trap the protein.

Using optical tweezers, we induced the mechanical unfolding of luciferase by stretching it with forces of up to 65 pN. We found that on the first pull, natively folded luciferase occasionally unfolded to the unfolded state (U; contour length $\approx$ 198 nm) in a single step with an unfolding force of about 40 pN (Figure 1b, blue curve). In most cases however, luciferase unfolding occurred through one to several unfolding intermediates, as evidenced by multiple unfolding steps (Figure 1b, green and black curves). Some unfolding intermediates were visited only briefly for tens of milliseconds, while others lasted up to several seconds. After stretching, the unfolded protein was relaxed and given the opportunity to refold at negligible applied force over a waiting time of 5 seconds. Subsequent stretching showed that the chain had either remained fully unfolded, become compact

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[**] This work was supported by the research programme of the Foundation for Fundamental Research on Matter (FOM), which is part of the Netherlands Organisation for Scientific Research (NWO).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201405566.

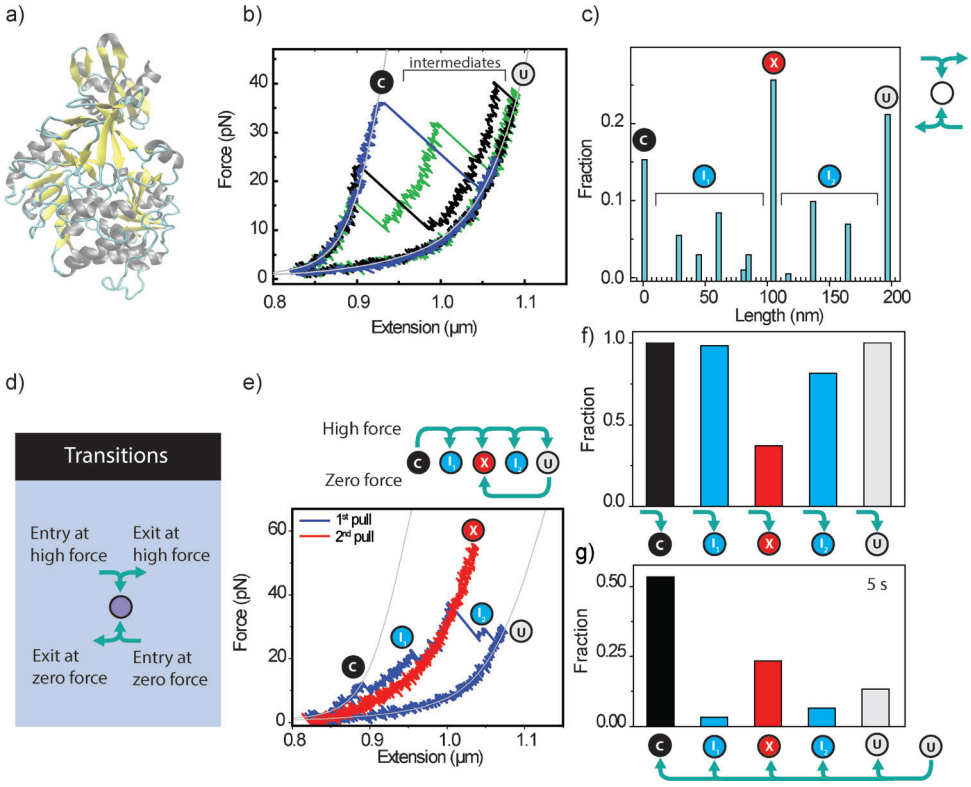

Figure 1. The unfolding and refolding of luciferase. a) The structure of Firefly luciferase (1LCI). Luciferase proteins were attached to micron-sized beads through a DNA linker.[18] b) Luciferase unfolding from the compact state (C) to the unfolded state (U). c) Visited protein lengths during stretching and relaxation cycles ($n = 203$). d) Different transitions that are observed in the experiments. e) Refolding from the unfolded state to the X state. f) Visited protein lengths during stretching ($n = 42$). g) Visited protein lengths during refolding ($n = 30$).

like the native state, or transitioned to something in between. In the latter two cases, stretching again resulted in unfolding.

To characterize the observed states, we quantified their apparent lengths by fitting the data to a worm-like chain model (Figure 1 b, gray lines). These lengths reflect the contour length of the non-folded part of the polypeptide because the folded part is compact and does not contribute significantly to length. Some intermediate lengths in between the most compact state (C) and the fully unfolded state (U) were visited more frequently (Figure 1 c). The most frequently populated intermediate state had a measured length of about 105 nm. We refer to this state as the X state. Intermediate states smaller than the X state were designated as the I1 states and those larger than the X state as the I2 states.

Next, we analyzed the probabilities for entering and exiting the different states during unfolding and refolding (Figure 1 d). In the measurement displayed in Figure 1 e, the polypeptide started in the compact C state and then sequentially visited the I1, X, I2, and U states during stretching. After relaxation and waiting at 0 pN, the polypeptide had a length similar to that of the X state, as seen from the subsequent pulling curve (Figure 1 e, red curve). In the subset of unfolding experiments that start in the C state and end up fully unfolded, entry into the X state occurs with a lower probability (p-value < 0.05) than entry into the I1 and I2 states (Figure 1 f). By contrast, in experiments where unfolded chains are relaxed and given the opportunity to refold at 0 pN, entry into the X state occurs with a higher probability (p-value < 0.05) than entry into the I1 and I2 states (Figure 1 g).

These observations led us to surmise that the X state could be a misfolded state. The rationale is that disrupting native-like states should not produce misfolded structures, which is consistent with the low probability of entry into the X state during unfolding. By contrast during refolding, unfolded chains can adopt misfolded structures, a fact that is consistent with the high probability of entry into the X state during refolding at 0 pN.

To test whether the X state is a kinetic trap, we investigated the folding kinetics. First, we considered refolding from the unfolded state. Increasing the waiting time at 0 pN from the 5 s used previously to 1 min decreased the probability of remaining unfolded down to zero (Figure 2 a). Such a decrease is consistent because the polypeptide now has more time to refold. At the same time, the I2 states were also no longer observed, thus suggesting that polypeptides that had adopted these states could continue to fold if provided with sufficient time. By contrast, the fraction of times the polypeptide was found to be in the X state had increased by about a factor of two. Even after waiting for 5 min, we still sometimes found the polypeptide chain in the X state (Figure 2 a). These observations agree with the idea that the polypeptide can become trapped in the X state. Note that entrapment in the X state is not obligatory since the protein chains often refolded to the compact state during the waiting period at 0 pN.

Next, we investigated exit from the X state. To achieve this, we first let the polypeptide fold into the X state by relaxing the unfolded state to 0 pN. When entry into the X state was confirmed by stretching to about 10 pN and measuring the protein length, the system was relaxed to 0 pN again before it could unfold. We found that after a subsequent waiting time of 5 s at 0 pN, the polypeptide had remained in the X state in the majority of cases (Figure 2 b). By contrast, when we similarly prepared the I1 and I2 states and relaxed

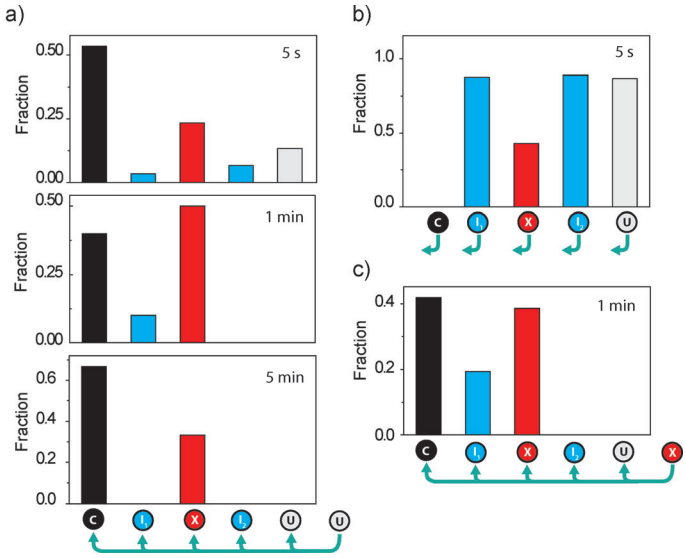

Figure 2. Entry into and exit from the X state. a) Entry into the X state at 0 pN after relaxing the unfolded state. The fraction of the experiments in which the polypeptide adopted a state with the indicated length is indicated. The different waiting times at 0 pN are indicated; $n=30$ (5 s), $n=10$ (1 min), $n=11$ (5 min). b) Exit from intermediate states at 0 pN ($n=61$). c) Exit from the X state after 1 min waiting time at zero force ($n=31$).

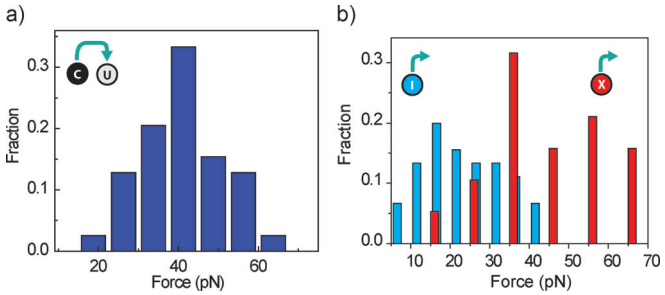

Figure 3. a) Measured C-state unfolding forces ($n=39$). b) Measured unfolding forces for the I1 and I2 states (blue; $n=45$) and the X state (red; $n=19$).

them to 0 pN, the polypeptide had exited these states after 5 s in the majority of the cases (Figure 2b). The exit probability was thus significantly lower (p-value <0.05) for the X state than for the other intermediate states. Increasing the waiting time to 1 min in these experiments increased the exit probability from the X state, but not by much (Figure 2c). In the cases where the polypeptide chain did exit from the X state, it was observed to have entered the I1 or C state, thus indicating that a larger part of the polypeptide had become folded. Overall, these data indicate that the X-state acted as a kinetic trap.

To estimate the stability of the X state to applied force, we performed forced unfolding experiments. We again prepared polypeptides in the X state by refolding as described above, and we then increased the applied force until the structure unfolded. As a comparison, we performed similar forced unfolding experiments for polypeptides prepared in the I1 and I2 states, as well as for the C state. We found that the unfolding forces for the I1 and I2 states were broadly distributed, with an average of 24.2 pN, while the C state unfolded on average at about 40.9 pN (Figure 3). The lower unfolding force for the I1 and I2 states compared to the C state can be understood from the fact that a smaller part of the polypeptide chain is folded and thus fewer intrachain contacts are formed. For the X state, we found that the unfolding force was 43.4 pN on average and was thus comparable to that for the C state but significantly higher than those for the the I1 and I2 states (p-value <0.05, Figure 3). These findings indicate that the X state, which like the I1 and I2 states has only part of the protein chain folded, is comparatively stable to forced unfolding. This higher relative stability is consistent with the low rate of escape from the X state at 0 pN when compared to the I1 and I2 states. The data show that over time, the X state can convert to the C state, while we do not observe the reverse, thus suggesting that the C state has a lower energy than the X state. However, we do stress that the unfolding force does not directly reflect thermodynamic stability. Other factors that affect the unfolding force are: 1) the relevant reaction coordinate when pulling a specific state and the height of the energy barriers along that coordinate and 2) the sensitivity to applied force, which is affected by the distance between the folded state and the unfolding transition state.[19]

Single-molecule approaches have opened up possibilities for the direct and real-time probing of folding transitions in biomolecules.[12b,17,20] Herein, we report single-molecule mechanical unfolding and refolding experiments on the multidomain protein firefly luciferase. Folding and misfolding transitions could be studied in the absence of mechanical tension by keeping the protein chains at negligible force for specified durations and then probing the new folded state by mechanical stretching. Since single luciferase proteins were interrogated, any confounding effects owing to interactions with aggregation partners were absent. The data show that during folding, relaxed luciferase protein chains can fold within seconds or become trapped for minutes and longer in a partially folded state. These results indicate that individual luciferase protein chains can misfold in the absence of (reversible) aggregation reactions[9] and can explain the experimentally observed inefficient refolding of luciferase.[15a] Misfolding provides an explanation for the tendency of luciferase to aggregate since long-lived partially folded states provide increased opportunities for aggregation. The ability to study misfolding transitions in individual protein chains is of importance for a range of questions. The assay presented herein could henceforth be used to study whether the transitions to and from the Luciferase misfolded state are affected by interactions with chaperones.

Received: May 26, 2014
Revised: June 30, 2014
Published online: ■■ ■■, ■■■■

Keywords: conformational transitions · misfolding · protein folding · protein models · single-molecule studies

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Zuschriften

Proteinfaltung

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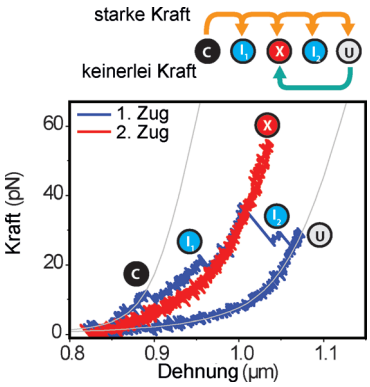
S. J. Tans* ——— ▮▮▮▮–▮▮▮▮

Misfolding of Luciferase at the Single-Molecule Level



Falsch gefaltet: Unter Anwendung einer Zugkraft entfaltet sich ein einzelnes Luciferase-Molekül in einem vielstufigen Prozess, wobei auf dem Weg zum vollständig entfalteten Zustand (U) mehrere intermediäre Konformationen durchlaufen werden (I). Wird der Zug gelockert, so kann das Molekül einen fehlgefalteten Zustand (X) mit langer Lebensdauer und hoher mechanischer Stabilität einnehmen, was die Bildung des komplett gefalteten Zustands (C) zurückdrängt.

Supporting information

EXPERIMENTAL PROCEDURES

Design, expression and purification of Luciferase

Avi-luci-4myc was expressed as hybrid protein consisting of an Ulp1-cleavable N-terminal His_{10}-SUMO tag followed by an AviTag, the luciferase gene and four consecutive myc-tags at the C-terminus. Overexpression was performed in *E. coli* BL21 cells harboring pBirAcm (Avidity, LCC, Aurora, Colorado, USA) in LB medium supplemented with 20 mg/l Biotin, 20 mg/l Kanamycin, 10 mg/l Chloramphenicol, 0.1 mM IPTG at 20°C for about 20 hours.
Cells from 1.5 l culture volume were lysed in buffer L containing 50 mM $NaPO_4$ pH 8, 0.3 M NaCl, 10% glycerol, 2 mM mercaptoethanol. The lysate was cleared from cell debris by centrifugation at 35.000 g for 30 min and incubated for 1 hour with 2 g Ni-IDA matrix (Protino; Macherey-Nagel, Düren, Germany). The matrix was washed extensively with buffer L and bound protein was eluted in buffer L containing 250 mM imidazol. Eluate fractions containing the hybrid protein were pooled, His_6-Ulp1 protease was added and dialyzed over night at 4°C in buffer L. The next day, the protein mixture was subjected to a second Ni-IDA purification to remove the His-tagged protease and the His_{10}-SUMO fragment and flow-through fractions containing purified Avi-luci-4myc were concentrated using Vivaspin concentration columns (Vivaproducts, Inc. Littleton, MA).

Optical tweezers measurement

For trapping, a Nd:YVO4 laser (Spectra Physics, λ=1064 nm, maximum power 5.4 W) was used [1, 2]. The trap stiffness in the pulling direction was 169±24 pN/µm for a 1.88 µm microsphere. Detection of forces on the trapped microsphere was performed using back focal plane interferometry. Forces were recorded at 50 Hz after application of an antialias filter at 20 Hz. During the experiments, a piezo-nanopositioning stage (Physik Instrumente) was used to move the sample cell and micropipette at a speed of 50 nm/s.

Anti-c-myc and anti-digoxigenin antibodies (Roche Diagnostics) were covalently coupled to carboxyl polystyrene beads (1.88 µm, Spherotech) using a carbodiimide crosslinking kit (Polysciences). To prevent unspecific binding of Luciferase to the polystyrene surface the antibody-coated beads were incubated with 1% (w/v) bovine serum albumin (BSA, Sigma Aldrich) and stored at 4°C until use. Luciferase-coated microspheres were made by mixing 4 µl Luciferase (0.5 mg/ml) and 2 µl anti-c-myc beads in 20 µl HMK (50 mM Hepes, pH 7.6, 100 mM KCl, 5 mM MgCl2) 0.1%BSA buffer. After 30 minutes incubation on a rotary mixer (4ºC), the beads were dissolved in 400 µl HMK/0.1%BSA buffer for use in optical tweezers experiments.

A dsDNA linker was produced by PCR using primers containing either two biotin or two digoxigenin groups (MWG-Biotech AG) and the plasmid pUC19 (New England BioLabs). The product of this PCR is a 2553bps dsDNA linker with two digoxigenin groups at the 5' end and two biotin groups on the 3' end. DNA-coated microspheres were made by first incubating 0.4 µg/ml streptavidin (Molecular Probes) with ~250 ng of the digoxigenin and biotinylated dsDNA linker in 10 µl HMK/0.1%BSA buffer for 15 minutes. Next, 2 µl anti-dig beads were diluted in 10 µl HMK/0.1%BSA buffer and mixed with the DNA/streptavidin solution. After 30 minutes on a rotary mixer (4°C), the polystyrene beads were resuspended in 400 µl HMK/0.1%BSA buffer for use in optical tweezers experiments.

We note that different folded states can display a similar measured length. For example, we do observe native partial folds during unfolding that have similar length as the X-state (though at low probability). Distinguishing these states from the X-state could be possible with high-resolution deconvolution process [3]. We also note that the unfolded protein may fold partially at low forces but before 0 pN is reached, which can result in an underestimation of the 5 s. folding time. In our experiments, the time spent at low force is less than a second, and hence the error in the folding time estimation is comparatively small.

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