



Ulrich Merkel

Single-Molecule Experiments on Enzymatic Peptidyl-Prolyl-Isomerization

Dissertation at the Physics Department E22
Technische Universität München

Single-Molecule Experiments on Enzymatic Peptidyl-Prolyl-Isomerization

**Controlling the folding kinetics of single proteins with
a new fast-force-jump single-molecule force spectroscopy
protocol for optical tweezers.**

Ulrich Merkel

Vollständiger Abdruck der von der Fakultät für Physik der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Martin Zacharias

Prüfer der Dissertation: 1. Prof. Dr. Matthias Rief
2. Prof. Dr. Andreas Bausch

Die Dissertation wurde am 13.02.2019 bei der Technischen Universität München eingereicht und durch die Fakultät für Physik am 22.04.2019 angenommen.

Abstract

Peptidyl-prolyl cis-trans isomerization is a regulatory mechanism in many biological processes. The intrinsically slow transition between the cis- and trans-state of proline is catalyzed by isomerases, a ubiquitous class of enzymes. It is unclear how isomerases influence the proline isomerization.

To investigate isomerase catalyzed proline isomerization, a custom-built high steering-speed, ultra-stable dual-beam optical trap was developed and subsequently used to perform a novel fast force-jump single-molecule force spectroscopy protocol. The fast force-jump protocol allows vast manipulation of protein folding behavior and an enormous temporal bandwidth, with millisecond resolution and stable experimental conditions over hours. A general model of the new fast force-jump experiment was developed to obtain individual rates for protein transitions.

The *Escherichia coli* protein SlyD is an example for an isomerase. SlyD catalyzed proline isomerization was studied on the human actin-crosslinker protein filamin A that shows native state cis-trans-heterogeneity. The proline that occurs in the cis- and trans-isoform is located in domain 20. The jump protocol was used to measure the force-dependent folding and unfolding rates of domain 20. In the cis-state, the folding rate was $k_{U_c, N_c}^0 = (185 \pm 40) \text{ s}^{-1}$ and $k_{N_c, U_c}^0 = (1.9 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$ for unfolding. The rates measured via the jump protocol are in good agreement with previous findings. To study the catalytic isomerization in domain 20, experiments were conducted in the presence of SlyD. Interestingly in the cis-configuration, neither folding nor unfolding was affected by SlyD. Contrary to expectations, no stable complex of SlyD and filamin A domain 20 was observed.

Furthermore, the jump experiments allow the observation of multiple proline cis-trans-isomerizations in a single protein for the first time. Thereby, the full kinetic network of domain 20 and its proline switch can be studied in detail. The apparent proline isomerization rates were found to be increased in the presence of rising SlyD concentration. The apparent catalytic efficiency of SlyD acting as an isomerase on the unfolded domain 20 was $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Contrary to previous assumptions, proline isomerization in the native state was found to be also catalyzed by SlyD. Thereby, the complex kinetic network of filamin A domain 20 was fully characterized in the presence of an isomerase for the first time. Notably, the enzymatic isomerization mechanism does not depend on a high binding energy of SlyD. In contrast, SlyD catalyzes proline isomerization via a transient interaction. The data provide detailed insight into SlyD catalyzed isomerization in domain 20, that underpin a preferential stabilization of the transition state. This work presents how advanced single-molecule force spectroscopy experiments can be used to gain a deeper understanding of proline isomerization, unveiling previously unexpected enzymatic isomerization pathways and the fundamental molecular mechanism.

Zusammenfassung

Prolin cis/trans-isomerisierung ist ein Regulationsmechanismus vieler biologischer Prozesse. Isomerasen, eine weit verbreitete Enzymklasse katalysieren die langsame Isomerisierung. Jedoch ist unklar auf welche Weise Isomerasen die Prolin-isomerisierung beeinflussen.

Um die Isomerase katalysierte Prolin-isomerisierung zu erforschen wurde im Rahmen dieser Arbeit eine schnelle, hochauflösende, ultrastabile optische Falle entwickelt und anschließend für Experimente mit einem neuartigen Einzelmolekülprotokoll schneller Kraftsprünge genutzt. Das Sprungprotokoll bietet enorme Möglichkeiten Proteinfaltung zu beeinflussen. Dabei ist die Bandbreite riesig, mit Millisekunden Auflösung und Messzeiten von Stunden. Es wurde ein allgemeines Model des Sprungprotokolls entwickelt, um einzelne Proteinübergangsraten zu messen.

Das *Escherichia coli* Protein SlyD ist eine Isomerase. Am humanen Aktin-Vernetzerprotein Filamin A, das cis/trans-Heterogenität im nativen Zustand aufweist, wurde SlyD katalysierte Isomerisierung untersucht. In Domäne 20 befindet sich ein Prolin, dass in cis- und trans-Form vorkommt. Mit Hilfe von Sprungexperimenten wurde die kraftabhängige Faltungsrate gemessen. In der cis-Form war die Faltungsrate $k_{Uc,Nc}^0 = (185 \pm 40) s^{-1}$ und die Entfaltungsrate $k_{Nc,Uc}^0 = (1.9 \pm 0.1) \times 10^{-2} s^{-1}$. Die Ergebnisse sind konsistent mit früheren Messungen. Zur Untersuchung der katalysierten Isomerisierung wurden Experimente an Domäne 20 in Gegenwart von SlyD durchgeführt. Interessant ist, dass in der cis-Form weder die Faltung noch Entfaltung durch SlyD beeinflusst wurden. Entgegen anderer Erwartungen wurde kein stabiler Komplex aus SlyD und Domäne 20 beobachtet.

Darüber hinaus erlauben Sprungexperimente erstmalig die Beobachtung mehrerer Prolin-isomerisierungen an einzelnen Molekülen. Damit kann das kinetische Netzwerk der Domäne 20 mit seinem Prolinschalter vollständig erforscht werden. Die anscheinenden Isomerisierungsraten waren mit steigender SlyD Konzentration beschleunigt. Die katalytische Effizienz von SlyD als Isomerase von Domäne 20 war $2 \times 10^6 M^{-1} s^{-1}$. Entgegen gängiger Annahmen, wurde auch die isomerisierung im nativen Zustand durch SlyD katalysiert. Erstmals wurde das kinetische Netzwerk von Domäne 20 in Gegenwart einer Isomerase vollständig charakterisiert. Interessant ist, dass der Mechanismus enzymatischer Isomerisierung nicht von einer hohen SlyD-Bindungsenergie abhängt. Im Gegenteil erwies sich die SlyD katalysierte Isomerisierung als transiente Wechselwirkung. Die Daten geben einen detaillierten Blick auf SlyD katalysierte Isomerisierung von Domäne 20 und stützen das Model der Stabilisierung des Übergangszustandes. Die vorliegende Arbeit zeigt wie fortschrittliche Einzelmolekül Kraftspektroskopieexperimente eingesetzt werden können um ein tieferes Verständnis von Prolin-isomerisierung zu erlangen, vorher unbekannte enzymatische Pfade zu entdecken sowie zugrundeliegende Mechanismen aufzuklären.

List of Publications

- GRISON M, MERKEL U, KOSTAN J, DJINOVIĆ-CARUGO K, RIEF M (2017) α -Actinin/titin interaction: A dynamic and mechanically stable cluster of bonds in the muscle Z-disk. *PNAS* 114: 1015–1020
- BAUER D, MEINHOLD S, JAKOB R P, STIGLER J, MERKEL U, MAIER T, RIEF M, ŽOLDÁK G (2018) A folding nucleus and minimal ATP binding domain of Hsp70 identified by single-molecule force spectroscopy. *PNAS* 115: 4666-4671
- SUREN T, RUTZ D, MÖSSMER P, MERKEL U, BUCHNER J, RIEF M (2018) Single-molecule force spectroscopy reveals folding steps associated with hormone binding and activation of the glucocorticoid receptor. *PNAS* 115: 11688-11693

Acknowledgments

I want to thank those people who contributed in various ways to this project over the years. First of all, I want to thank my supervisor Prof. Dr. Matthias Rief for many great ideas and that he made this work possible in the first place. His door was always open to discuss data or tackle tricky problems with the setup or equipment. The great freedom for original ideas and new approaches, paired with the highest standard for results have contributed to my scientific development.

I thank Dr. Gabriel Žoldák for practical guidance and advice in scientific day-to-day business. I appreciate the many fruitful discussions, constructive feedback, encouragement and support from him.

I would like to acknowledge Dr. Soumit Mandal for direct contribution to this project by sharing valuable data with me. Lorenz Rognoni introduced me to the project and provided the constructs.

Daniela Bauer saved me in countless situations where I was fighting biochemistry's beginner's mistakes. With Benjamin Pelz, Heinrich Grabmayr and Matthias Schuppler I shared the joy and frustration of building a complex optical setup with great, as I hope mutual, benefit. Johannes Stigler and Markus Jahn helped me developing new and using the e22 written software. A number of people always helped out with personal support, fruitful discussions, hands-on contributions or inspiring ideas: Alexander Mehlich, Andreas Weißl, Fabian Ziegler, Johannes Stigler, Katarzyna Tych and Marco Grison. I am thankful to everybody who helped me in the complex interplay between physics, biochemistry, and biology.

I want to thank everybody in the bio corner of the physics building and all the members of the Rief, Bausch, Dietz, Hugel and Woehlke labs for the great fun and their support.

I am thankful to my family for their encouragement and support. Finally, I want to thank my girlfriend for her great, enduring support.

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Part I

Introduction

1. Protein Folding

Life's fundamental functions such as metabolism, molecular transport signal transduction, or movement are generated by proteins. The organism's proteome provides the machinery that uses energy to help create, maintain and control cell structure and function. There is an alphabet of 21 proteino-genic amino acids that covalently link via peptide bonds one after another. Proteins consist of one-dimensional amino acid chains that mostly gain their function by folding into a specific three-dimensional structure. This structure is encoded in the amino acid sequence.

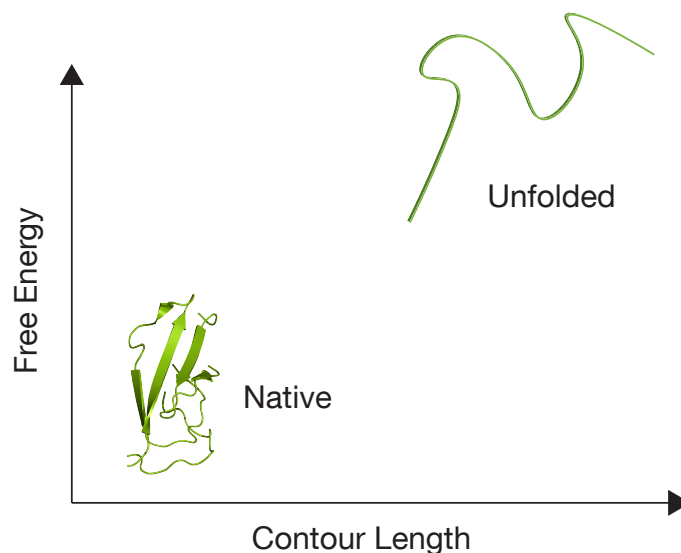


Figure 1 Free Energy of a protein in the native and fully unfolded state. The native state is at lower free energy. The extension of the peptide chain, the so-called contour length as reaction coordinate is bigger for the unfolded state.

Figure 1 shows the conformation-dependent free energy of a protein in its solvent. This so-called energy landscape determines the protein's folding properties. The functional, native state of a protein is typically a conformation that has minimal free energy. Nevertheless, a general mechanism by which proteins are folding is still not known. However, an unbiased search in the conformational space of a big unfolded protein would take an eternity to spontaneously find and click into its native state [Lev68]. One way to bring the folding times from astronomical down to feasible is to assume a funneled energy landscape and thereby, conformational preferences towards the native state [FGB96].

Figure 2 shows a funnel-shaped energy landscape of a protein that populates partly folded, so-called intermediate states. Stable conformational

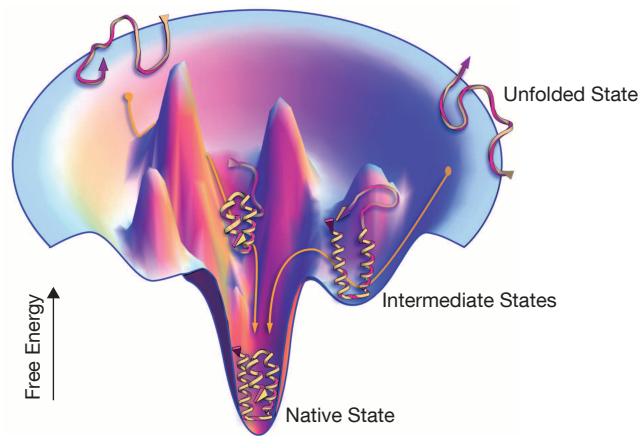


Figure 2 Protein folding in a funnel-shaped energy landscape. The unfolded peptide chain is driven towards the native state, following the free energy gradient. Local minima in the energy landscape cause intermediate states. Adapted from [DMF12]

states can only be found at minima in the energy landscape. Typically, well defined structural motifs form in a cooperative manner, into these intermediate states that can be mandatory, *on-pathway* for folding. All populated states, the protein adopts during the folding process form a kinetic network. Transitions from one protein state to another can be seen as diffusion in the energy landscape. In a simple model, the free energy difference of states relates to their population probabilities and the energy barrier height to the respective transition rates. Depending on the height of the free energy barrier that has to be overcome between distinct states, the direct transition is more or less likely. Partly folded, or miss-folded states can form kinetic traps that considerably slow down folding [BHB75]. In Summary, the mechanism on which proteins fold is still not fully understood. The current model of the folding funnel describes the observed folding processes of single proteins well.

2. Peptidyl-Prolyl Cis-Trans-Isomerization

petence of the protein with the proline in the non-native isoform. Although local displacements caused by proline isomerization are minor, it eventually leads to rearrangement of large structural parts. Proline isomerization of the protein backbone is involved in the regulation of many biological processes: such as molecular timers [LFLN07], switches [And03] or force sensors [RMOR14]. A special example is the proline isomerization-induced structural changes, that cause opening and closing of ion channel pores [LBL⁺05].

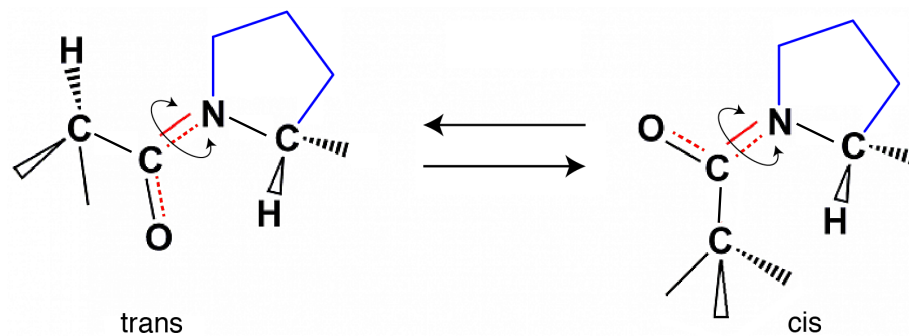


Figure 4 Proline bonds, like all peptide bonds, are partial double bonds (red). The rotation around the N-C bond is therefore restricted. Unique to proline is a second connection of the side chain to the backbone, forming a ring structure (blue). Noteworthy, only proline bonds populate the cis isoform considerably, due to steric effects of the ring. In unfolded proteins, the cis and trans isoform interchange on a timescale of minutes.

Figure 4 illustrates the picture that proline isomerization is a 180° rotation of the peptide bond. Due to the partial double bond character of peptide bonds, the energy barrier between the cis- and trans-states is high and thus the transition kinetic is slow (on the timescale of minutes) [CB77], [BHB75]. In contrast, intracellular correlation times are typically shorter than seconds. Taken together, beyond its importance for protein folding, proline isomerization is an intrinsically slow, molecular regulation mechanism for different functional protein conformations.

2.1. Peptidyl-Prolyl Isomerases

Enzymes are molecules that catalyze specific chemical reactions, in these reactions, they convert their substrates into the products. In most living organisms peptidyl-prolyl cis-trans isomerases (PPIases) are important enzymes, catalyzing up to 4% of the chemical reactions in the central metabolism [MFR⁺14]. PPIase activity was first studied via their ability to accelerate proline isomerization limited protein folding [Jae93]. Nature has evolved four enzyme families of PPIases: FK506-binding proteins (FKBPs), cyclophilins, parvulins, and the protein Ser/Thr phosphatase 2A (PP2A) activator PTPA. These PPIases catalyze proline isomerization. PPIases have been the subject of intense research because they have been implicated in many diseases. For example, family members of FKBP are

cellular targets during immune suppression after organ transplantation by FK506. However, PPlase activity turned out not to be the mechanism of immune suppression [SC92]. The FK506/FKBP complex specifically inhibits calcineurin, resulting in a failure to activate genes necessary for T-cell proliferation [HCT⁺96]. Another example, rapamycin is used in immune suppression and as an anticancer agent [HGUS89], [SHP⁺89]. In fact, immune suppression by FK506 and rapamycin is due to inhibition of evolutionarily conserved signal transduction pathways. FK506, and rapamycin bind to their intracellular receptors, immunophilins, creating composite surfaces that block the activity of specific targets.

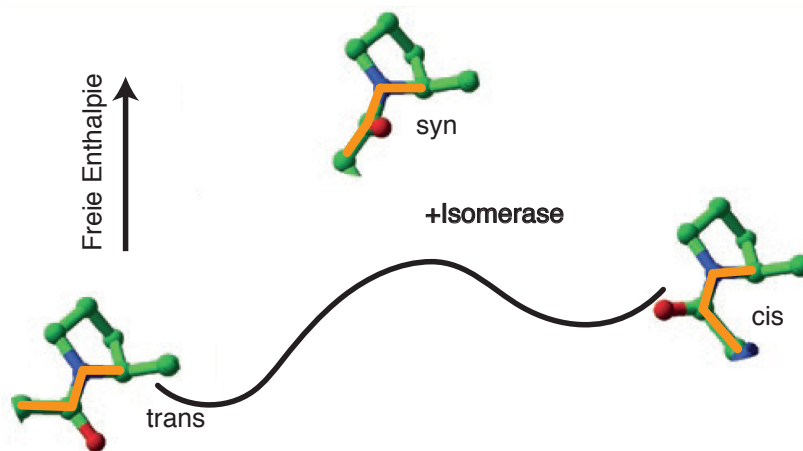


Figure 5 Peptidyl-prolyl cis-trans isomerization scheme. Ring formation due to binding of the side chain to the backbone is unique to proline bonds and stabilizes the cis-state. Due to the large conformational change inherent to the 180° rotation of the backbone, the energy barrier between the cis and the trans isoform is high (blue line). Enzymes of the PPlase subclass catalyze the proline isomerization by an, yet undefined molecular mechanism. Two proposed mechanisms are catalysis by distortion — binding of the enzyme induces a twist, distorting the planarity of the proline bond — effectively destabilizing the ground state (yellow line) and secondly, transition state stabilization (red line). Adopted from [LFLN07].

Not only the action of PPlases are subject to research, but also their detailed molecular mechanism. Figure 5 shows a cartoon representation of proline isomerization and corresponding energy landscape models. Of the two planar states trans and cis, the former is energetically favorable. In the transition- or so-called syn-state, the proline bond is rotated by 90° with respect to the other states. A general mechanism of how these enzymes catalyze proline isomerization has not been found after decades of studies. However, controversies on the true catalytic mechanism driving proline isomerization are still prevalent [FF04], [AMD⁺12], [HM09]. Today, mostly two mechanisms of enzymatic proline isomerization are considered [TSBH15]. One that is based on the lowering of the energy barrier by ground state destabilization (catalysis by distortion, yellow line in figure 5) [RSG⁺90] and another that only depends on twisted state stabilization (syn state, red line in figure 5) [PAF⁺92].

In many enzymes, distinct domains mediate the interaction with a folding

protein chain and the catalysis of intrinsically slow reactions. A particularly well-studied example of a PPlase is the two domain sensitive-to-lysis protein D (SlyD) of the FKBP family. SlyD proteins are highly conserved across different species, can bind up to seven Nickel ions and show chaperone as well as high isomerase activity [SEH⁺06], [LNT⁺10], [KSB13].

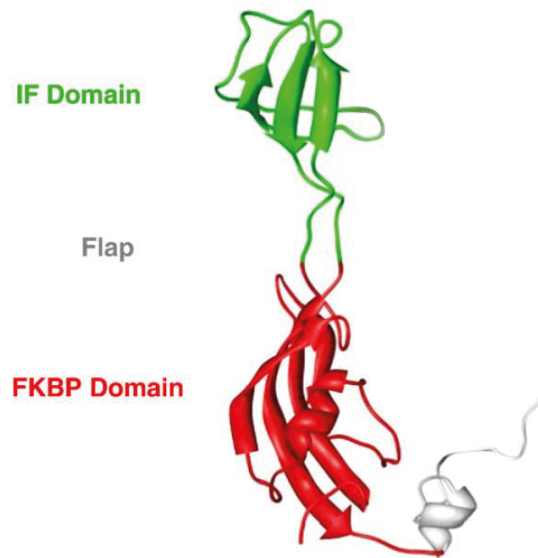


Figure 6 Crystal structure of a shortened SlyD (residues 1-165) version lacking the unstructured cysteine-rich and histidine-rich C-terminal tail. The tail is non-essential for PPlase activity or peptide binding. The IF-domain (residues 70-129) is shown in green, the C-terminal part (147-165) in gray and the FKBP domain in red. Adapted from [ŽCSS09].

Figure 6 shows the molecular structure of the 25 kDa SlyD, found in *Escherichia coli*. The main structural elements of SlyD are the 90 residues isomerase domain (shown in red in figure 6), the 60 residues chaperone domain (shown in green) and a C-terminal part (shown in gray) [WHS⁺09]. The two well-structured domains of SlyD are not arranged successively along the protein chain. SlyD's isomerase domain is a structural homolog to human FKBP12 and for this reason called FKBP-domain. In FKBP12 the β -strands two and three are connected by a loop, called "flap". The chaperone domain of SlyD is inserted between these β -strands of the isomerase domain, therefore called inserted into flap (IF)-domain. The two domains in SlyD are connected by a flexible linker, resulting in a highly dynamic relative position [KKL⁺11].

In proline isomerization limited folding, SlyD acts as an effective PPlase on proline bonds [ŽGS13]. Unfolded polypeptides bind to the IF-domain with high affinity [SEH⁺06], [ŽS11]. Binding and release of a permanently unfolded target peptide were found to be highly dynamic with a 40 ms complex lifetime [ŽS11]. Within the lifetime of the complex, the FKBP-domain with the catalytic center samples many positions in close proximity to the target peptide. Moreover, a single FKBP-domain has PPlase activity on short peptides [KES⁺07]. SlyD shows high PPlase activity on proline at position

2225 in filamin A (p2225) in domain 20 [Rog14]. How this PPlase mechanism works is still unknown. A model for enzymatic proline isomerization in two consecutive steps was proposed. Here in a first step, SlyD binds the substrate and then efficiently catalyzes proline isomerization in the bound state. While SlyD is bound, the proline bond undergoes constant back and forth isomerizations. After unbinding of SlyD, the proline bond is left in whatever isomerization state it was in the moment of unbinding.

2.2. Experiments to Observe Enzymatic Proline Isomerization

Kinetic networks of proteins that involve proline isomerization are typically dynamic and complex. Kinetic networks that involve proline isomerization potentially include parallel pathways between the states in the presence of a PPlase. To experimentally unveil the mechanism of enzymatic proline isomerization it is necessary to be able to distinguish PPlase bound and unbound states. PPlases are known to speed up the proline isomerization kinetics by orders of magnitude. In the unfolded protein, the enzymatic proline isomerization rates can reach 100 s^{-1} [ŽGS13], whereas native state proline isomerization rates may be $5.5 \times 10^{-5} \text{ s}^{-1}$ [RMOR14]. Thus, a suitable experimental protocol must provide an enormous temporal bandwidth.

Established methods cannot distinguish the isoforms of proline bonds with high temporal resolution. Thus a detailed molecular understanding of enzymatic proline isomerization mechanisms is still lacking. However, it might be similar among the different PPlase families [FF04]. Various methods have been used to study proline isomerization. One example is to use fast mixing techniques to study proline isomerization in unfolded peptide chains. Another example is to use isomer specific protolysis experiments. During folding experiments on proteins with isomer dependent folding, a double exponential time distribution is observed. This can be explained by peptide chains with the proline bond in the correct, specific isoform folding fast, while the slow proline isomerization is the rate-limiting step. Due to enzymatic proline isomerization in the presence of a PPlase, the slow phase vanishes [FB85]. Nevertheless, cis-trans-isomer heterogeneity in folded proteins is not observable.

However, cis-trans coexistence in the native state of proteins is observable using *nuclear magnetic resonance spectroscopy* (NMR) methods, if both isomers are populated considerably [EDK⁺87]. NMR is a technique to observe the cis- or trans-isoform of peptide bonds, as well as the intrinsically slow non-enzymatic transitions and has been used for decades e.g. [SS70], [KKS⁺95], [WRJ10]. Furthermore, isomer dependent oligomerization of small heat-shock proteins, and thus native state proline isomer-

ization can be observed with NMR [ABB17]. Nevertheless, proline conformation and dynamics are not well resolved by NMR spectroscopy. Proline isomerization is effectively catalyzed by PPlases, thus distinct signals from *cis*- and *trans*-states average within the NMR timescale, that is about 1.5 s [BFN⁺07]. Thus, it appears non-trivial to study enzymatic proline isomerization. Established single-molecule force spectroscopy (SMFS) protocols using optical tweezers have been used to observe the *cis*- and *trans*-isoform of a proline bond in a folded protein domain under mechanical force [RMOR14]. The equilibrium un/-folding kinetics unveil intrinsic transition rates of non-enzymatic *cis-trans*-isomerization. The *cis-trans*-isomerization measurement depends on the folded state, thus temporal resolution is limited by the folding rate against force, often on the timescale of seconds [RMOR14].

Active triggering of protein folding by force-jump can be used to enable SMFS studies on enzymatic proline isomerization. In this work a new *fast force-jump* protocol is presented, a double-jump stopped flow inspired advanced single-molecule force spectroscopy technique. A custom-made high-resolution optical tweezers setup was built and used to drive protein un/-folding far out-of-equilibrium to overcome the temporal resolution limits. This powerful protocol enables SMFS enzymatic proline isomerization studies at the single-molecule level.

3. The Filamin Protein Family

Members of the filamin family are large actin-binding proteins. The V-shaped homo-dimers play important roles in cross-linking actin filaments to build a dynamic three-dimensional structure: the cytoskeleton. The name filamin refers to its filamentous co-localization with actin stress fibers. Filamins are highly conserved and share characteristic repeated β -sheet units (figure 7).

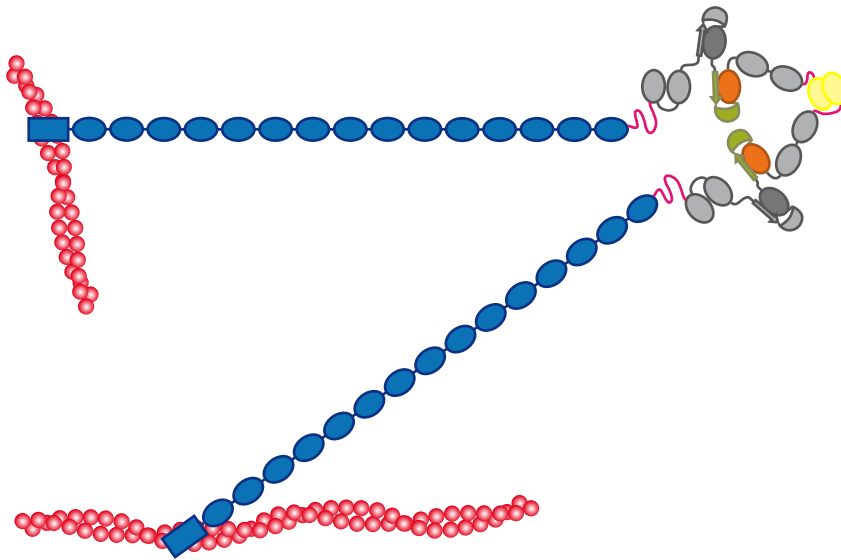


Figure 7 Domain arrangement of one filamin A homo-dimer cross-linking two actin filaments (red). The N-terminal actin-binding domain (blue rectangles) is followed by 24 β -sheet repeats. The monomers dimerize at domain 24 (yellow). The repeats 1 to 15 (blue ellipses) form the rod 1 region and 16 to 23 (gray, orange and green ellipses) rod 2 with subsequent flexible hinges (pink). In rod 2, the domains 16 to 21 arrange in pairs that contain binding sites for transmembrane proteins. The force-sensing domain pair 20–21 is highlighted in green and orange. Adapted from [RMOR14].

3.1. Filamin A Structure

Filamin A binds and cross-links actin through its N-terminal actin-binding domain and dimerizes at its C-terminal domain. Two flexible hinges divide the filamin A monomer into three major domains: the rod 1 region containing repeats 1 to 15, rod 2 is formed by repeats 16 to 23 and the C-terminal dimerization domain. The major partner-interaction domains are located in the rod 2 region. Two examples for interaction partners are the transmembrane proteins integrin and glycoprotein Ib (GPIb) [Uta09], [JC08], [RMOR14]. The cytoskeleton is linked to the extracellular matrix via trans-

membrane proteins that bind to filamin A domain 21. The binding affinity of the transmembrane proteins is controlled by a force-sensor based on a proline-switch in domain 20. The AB-strand of domain 20 forms a β -sheet that binds to domain 21 and thus inhibits the transmembrane protein binding side there.

The force-sensing proline-switch controls transmembrane protein binding. Domain 20 of filamin A (FLNa20) alone can fold into two distinct native states, with the p2225 being either in the *cis*- or *trans*-isoform. The folding-free energy of the native state with the proline in *trans*-conformation is smaller. Previously, the un-/folding kinetics have been used to discriminate between these two conformations. The proline isomerization kinetics of p2225 has been studied using SMFS [RMOR14]. Under mechanical stress, the binding side opens and transmembrane proteins can stably bind. In the open conformation, the native *cis*-p2225 located in a loop is likely to isomerize to *trans*. Thereby the inhibited conformation gets destabilized and keeps the binding side open for minutes. Thus, the binding affinity of transmembrane proteins increases sevenfold [RMOR14]. The location of p2225 in a loop has two noteworthy consequences: in the native state, the proline bond is shielded from mechanical strain and it is potentially accessible to PPlases.

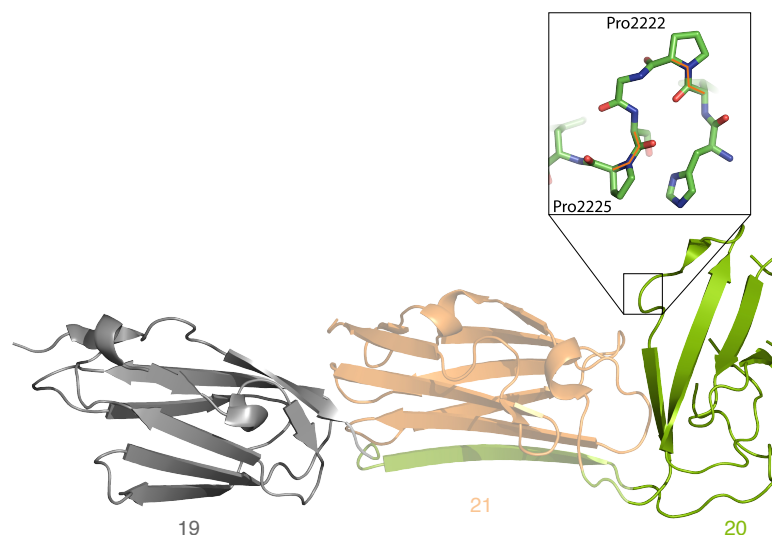


Figure 8 Crystal structure of human filamin A domains 19 to 21. A β -strand of domain 20 (shown in faded green) binds to a binding site in domain 21 (shown in orange) inhibiting binding of transmembrane proteins. Force-induced *cis-trans*-isomerization of p2225 revokes the auto-inhibition. P2225 in the bright green part of FLNa20 undergoes proline isomerization. The isomerization state governs the un-/folding kinetics of the bright green structure which is subject of investigation in this work. The proline at residue 2222 is in the more common *trans*-isoform. Adapted from [LKJ⁺07].

3.2. Kinetic Network of Filamin A Domain 20

FLNa20 lacking the β -strand that auto-inhibits the integrin binding-side in domain 21 (bright green in figure 8), acts as a two-state folder. Noteworthy, the native-state stability during SMFS experiments is dependent on the p2225 cis-trans-isoform. Due to FLNa20 bimodal stability, the isoform of p2225 can be monitored during SMFS experiments. All experiments presented in this work are conducted on a FLNa20 construct that lacks the initial β -strand, the exact protein sequence is shown in Appendix A.2. The single domain FLNa20 construct populates four distinct states:

- unfolded peptide of FLNa20 with p2225 being cis (U_c),
- native state of FLNa20 with p2225 being cis (N_c),
- unfolded peptide of FLNa20 with p2225 being trans (U_t),
- and native state of FLNa20 with p2225 being trans (N_t).

The different contour lengths of the folded and unfolded states are observable during SMFS experiments. Un-/folding lifetimes during passive mode experiments are on the timescale of seconds, whereas the isomerization correlation time is in the minute's regime. Thus, it is possible to assign cis and trans phases by their long-lived and short-lived native states during passive mode SMFS experiments [RMOR14]. SlyD catalyzes proline isomerization in the force-sensing domain FLNa20. The molecular interaction mechanism of SlyD and FLNa20 is still unknown. However, the underlying complex kinetic network of states is expected to contain various proline isomerization pathways and possible bound states. Figure 9 shows a proposed model of the kinetic network.

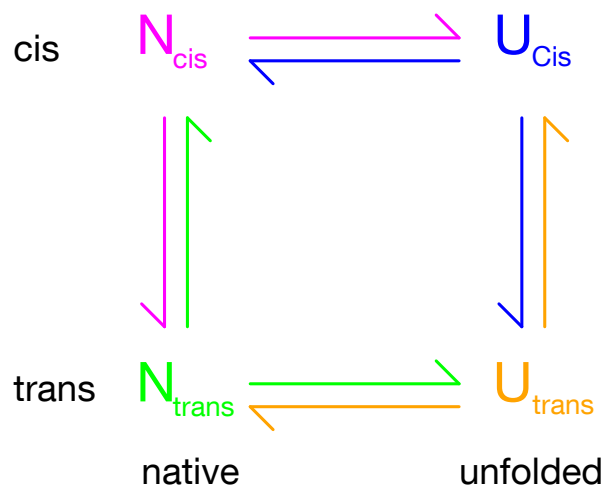


Figure 9 The kinetic network consisting of the folded, unfolded, cis- and trans-FLNa20 states.

3.3. Transition Dynamics of FLNa20 in the Presence of Peptidyl-Prolyl Isomerases

Proline isomerization catalyzed by SlyD shows rates near 100 s^{-1} [ŽGS13]. Since un-/folding kinetics are on the timescale of seconds are used as a readout during equilibrium SMFS experiments, the cis- and trans-states of p2225 are no longer resolved. The analysis of non-equilibrium experiments requires the proper assignment of all states at all time. Enzymatic back and forth isomerization can occur faster than the typical temporal resolution of established non-equilibrium experiments. Even more, SlyD is known to bind unfolded peptide chains [ŽGS13]. The complex kinetic network of FLNa20 in the presence of SlyD may contain bound states and enzymatic transition paths. Above all, the enzymatic proline isomerization rates provide no direct readout and are expected to be faster than un-/folding in equilibrium.

Part II

Methods and Theory

4. General Introduction to Single-Molecule Force Spectroscopy Experiments with Optical Tweezers

Acceleration and stable trapping of particles in a focused light amplification by stimulated emission of radiation (LASER) beam was reported for the first time in 1970. Back then, it was estimated that reflected light of a focused 1 W argon LASER source on a dielectric bead exerts a force in the order of 100 pN [Ash70]. These instruments were used to separate different isotopes of the same atom. A single-beam gradient force optical trap (optical tweezers) capable of trapping dielectric particles in a three-dimensional potential as proposed in 1978 [Ash78] was realized in 1986 [ADBC86]. Particles trapped by optical tweezers can be manipulated and the force acting on them can be measured in three dimensions.

Meanwhile, optical tweezers were used to study various biological questions, e.g. transcription of ribonucleic acid (RNA) [YWS⁺95] and biological systems that generate forces in vivo, such as molecular motors [BMpK⁺10]. First SMFS studies on protein un-/folding with optical tweezers have been reported in 2005 [CSBM05]. Therefore, the protein is coupled to micrometer-sized beads that are trapped in steerable optical tweezers. If dual-beam optical tweezers created from a single LASER source are used to exert a force on the protein the force resolution is limited by Brownian noise [MCIB06]. SMFS is a method to manipulate and observe a single protein moving in its conformational space under force. Many proteins can populate more than one stable state creating a kinetic network of states. A consecutive series of different states starting in the completely stretched state of the peptide chain and ending in the native folded state is called a folding *pathway*. SMFS experiments with optical tweezers are used to unveil protein un-/folding *pathways* [BMP⁺15] or the molecular mechanism of ligand binding [MMB⁺17], [RSP⁺12]. In summary, a dual-beam optical tweezers instrument provides stable experimental conditions for hours. At the same time, it is possible to observe protein un-/folding transitions on a timescale of milliseconds at physiological relevant forces of $\approx 3 - 30$ pN.

4.1. The Principle of Optical Trapping

Optical tweezers are widely used to trap small particles and manipulate them with high accuracy. A number of review articles give a broad overview

of the details on optical trapping, see e.g. Ref. [Blo92], [MJG⁺13], [Sha06] and [SB94]. There are three specific models for the different wavelength regimes. The optical trapping principle for objects larger than the LASER wavelength can be visualized with simple ray optics [Lor81]. Wave optics describe the interaction of light with objects that are small compared to the light wavelength. In SMFS experiments, the trapped objects are spherical dielectric beads with a diameter approximately equal to the wavelength of the LASER beam forming the optical trap. For such objects wave optical effects and light-matter interaction cannot be neglected. Theoretical description of the interaction of light with spherical objects in this regime is based on the classical *Mie theory* [Mie08]. A detailed theory of optical trapping can be found in Ref. [RS02], an optical tweezers computational toolbox is available for MATLAB [NLS⁺07].

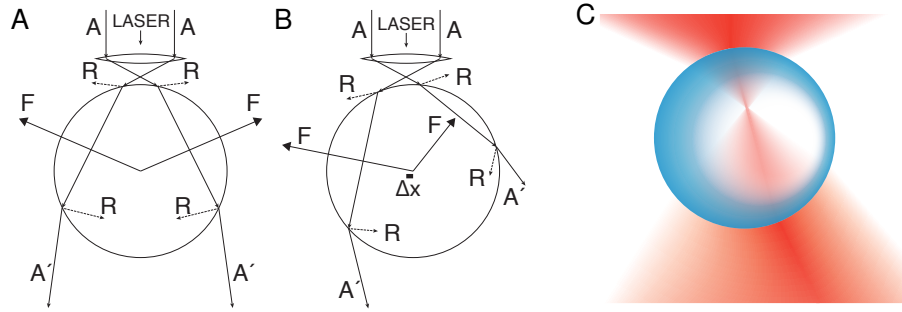


Figure 10 A typical pair of rays A of a highly focused LASER on a dielectric sphere in a stable trapping position (A). The major contribution to momentum transfer to the bead is due to refraction of emergent rays A'. This focusing causes a force pointing towards the LASER focus. Surface reflections R contribute to LASER scattering. The resulting forces F add up to a net backward trapping force toward the beam focus. The refraction and reflection angles change drastically if the bead is deflected a distance x from its stable trapping position (B). Schematic representation of a trapped bead that is out of the LASER focus (C). The bead is displaced to the right of the LASER focus. The beam is deflected to the right, thus the total force exerted on the bead points to the left. (A) and (B) adapted from [ADBC86].

Figure 10 shows a simple ray optics cartoon of a dielectric, spherical object —representing a silica bead typically used in SMFS—trapped in optical tweezers. The trapping potential is created from a LASER TEM 0,0 mode focused by a microscope objective with a high numerical aperture (NA). In the stable trapping position of the bead, light is refracted on the bead. The change of the light momentum counterbalances the light pressure from reflection on the bead surface (figure 10A).

If the bead is displaced out of the optical axis, the LASER beam is additionally deflected to the same direction (figure 10B). The change of the light momentum exerts a restoring force on the deflected bead. The Gaussian beam profile of the LASER creates a trapping potential that is in good approximation harmonic for small displacements Δx . The trap thus acts as a Hookian spring on the trapped bead exerting a restoring force

$$F = -\kappa \Delta x \quad (4.1)$$

on the bead¹. All displacements observed during experiments presented throughout this work are small.

4.2. Force Exertion on a Protein in a Dumbbell Configuration

The objects that are manipulated via optical tweezers in SMFS experiments are spherical silica beads with a diameter of $0.5\text{--}1\text{ }\mu\text{m}$. Notably, beads must be in the order of 100 nm apart, to observe well separated trapping potentials. A common optical tweezers SMFS configuration is the use of deoxyribonucleic acid (DNA) molecules as so-called *handles* to separate the investigated protein from the bead surface and the trapping LASER beams. Additionally, this strategy reduces photo-physical interaction and heating due to LASER absorption and undesired surface reactions that influence or damage the protein.

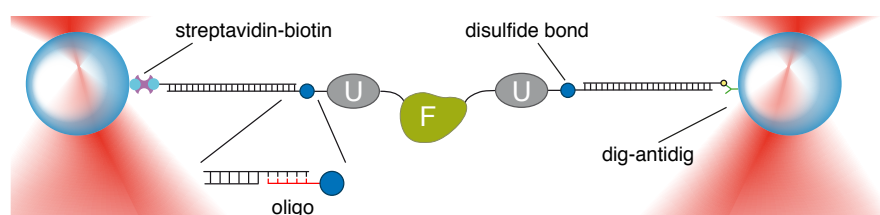


Figure 11 Cartoon representation of the dumbbell configuration. The sample protein (green) with two flanking ubiquitin domains (gray) is attached to silica beads (transparent blue spheres) via functionalized double-stranded DNA linker molecules (black ladders). The linkers are attached to the beads by an Avidin – Biotin / Digoxigenin – Antidigoxigenin interaction. A single-stranded DNA overhang at the end of the linkers can hybridize to single-stranded DNA-oligonucleotides (red) that were previously linked to the protein.

The *bead-DNA-protein-DNA-bead* construct, as shown in figure 11 is named after the appearance of the stretched system: *dumbbell configuration*. A multi-step protocol was used to assemble the dumbbell construct with high reaction efficiency and thus experimental yield. Various bioconjugation techniques and a selection strategy to attach the DNA handles to the protein can be found in Ref. [SF11].

In the following, the dumbbell configuration assembly shall be explained, starting with the protein: molecular cloning techniques were used to replace all internal cysteines with serines and insert the single protein domain between ubiquitin domains with terminal cysteines. For more details see Appendix A.2. The ubiquitin domains facilitate recombinant expression and purification and serve as spacers to prevent disulfide bridge formation between the terminal cysteines. The ubiquitin domains do not unfold under

¹ Due to this proportionality the physical quantities bead deflection and force exerted on the sample is used interchangeably. Trap distance in many scenarios is proportional as well.

forces applied during an optical tweezers experiment and thus do not interfere with SMFS experiments [SLF04]. To attach the DNA oligonucleotides to the sample protein, a strategy based on Strain-Promoted Azide-Alkyne (copper free) Click Chemistry (SPAAC) was applied, as sketched in figure 12.

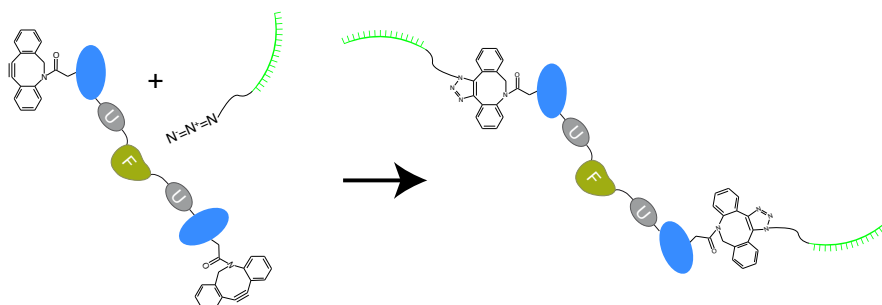


Figure 12 Principle of SPAAC based oligonucleotide attachment. In a first reaction, the DBCO-functionalized maleimides bind to the exposed terminal cysteines of the protein construct (not shown). DBCO is shown in structural formula representation, the maleimide group in blue and the sample protein in gray and green. In a second step — the click reaction — Azide-functionalized oligonucleotides (ssDNA shown in green) form a cross-link via triazole moiety.

In a first step, dibenzocyclooctin (DBCO)-functionalized² maleimides react with the thiol group of the protein constructs terminal cysteines. This reaction happens fast, on the timescale of minutes, thus preventing construct dimer formation via the terminal cysteines. Azide-functionalized DNA oligonucleotides can bind to the construct (for more details see Appendix A.3).

The beads are silica spheres with 1 μm diameter and surface amino groups. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ N-hydroxysuccinimide (NHS) cross-linking was used to coat the beads with either streptavidin or antidigoxigenin. The construct was preincubated with streptavidin-functionalized beads. Dumbbell assembly under the microscope is realized when the construct carrying beads are brought in close proximity to antidigoxigenin-functionalized beads.

4.3. The Optical Tweezers Instrument Designed and Built for this Work

The transition kinetics in complex kinetic networks of proteins can be fast and the corresponding signal in SMFS experiments can be small. In SMFS experiments a force is exerted on the protein of interest on specified positions to probe its state. In the force regime of several piconewtons the optical tweezers give high resolution, thus small changes of the protein state's free energy are resolved. Typically, two *single-beam gradient force*

² Dibenzocyclooctin is one of the most reactive cycloalkynes used for strain promoted alkyne azide cycloaddition

traps are created from one LASER source, providing stable experimental conditions over hours [MCIB06].

Therefore, a custom-built optical tweezers setup was designed and built for this work. Various considerations concerning drift, stability, and performance have been taken into account (see section 4.3.1). A small overall footprint facilitates extending the setup with fluorescence assays on the remaining space of the optical table. Two dichroic mirrors flanking the microscope objectives can be used to couple a fluorescence system with the optical tweezers.

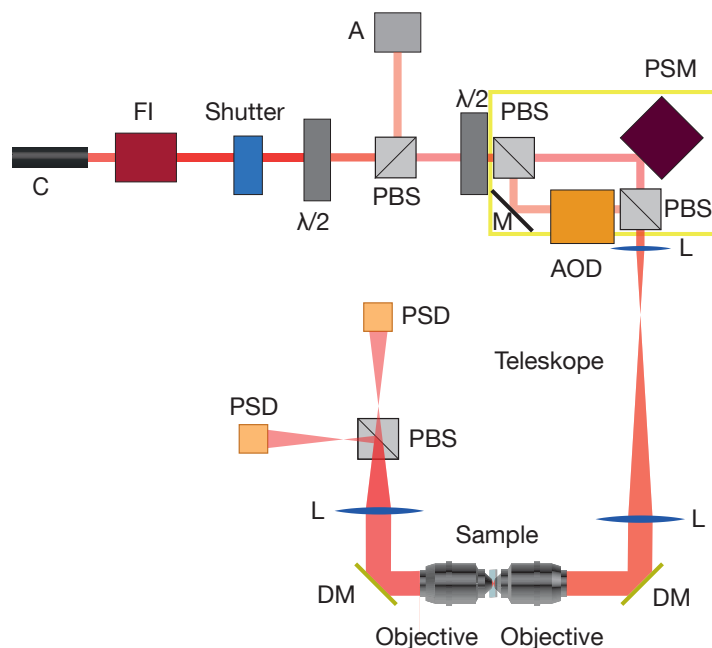


Figure 13 Sketch of the dual-beam optical tweezers design. The optical tweezers are created from the S- and P-polarized fractions of a linearly polarized fiber laser. The yellow rectangle indicates where the beam paths do not overlay. PSD: position sensitive device, DM: dichroic mirror, FI: Faraday isolator, PBS: polarizing beam splitter, A: absorber, PSM: piezo scanning mirror, AOD: acousto optical deflector, M: mirror, C: collimator of a 1064 nm LASER, L: lens

Figure 13 shows a schematic of the setup. An optical tweezers instrument providing stable experimental conditions for hours is needed. At the same time, it is necessary to observe FLNa20 folding and unfolding on a timescale of tens of milliseconds.

4.3.1. Stability and Trap Steering Speed Considerations

A high resolution, low drift optical tweezers setup has been designed and built before [VMP⁺12]. From this existing design, basic elements were adopted, but enhanced stability and trap steering speed could be achieved within the presented work by implementing additional considerations beyond the

ones summarized in Ref. [Pel14]. The use of acousto optical deflectors (AODs) ³ for high-speed trap steering is an established approach. The steering speed is limited by the response of the bead damped by the viscous drag of the watery surrounding. The high trap steering speed can be used for time shared multiplexing, where the trap position is cycled fast between multiple trapping positions. In the multiplexing mode, the optical tweezers off-time is shorter than the trapped particle's diffusion time out of the trapping potential. In force jump protocols high steering speed is crucial (chapter 7).

Major drift sources are the pointing and power instabilities of the typically used diode-pumped-solid-state LASERs sources. Pointing instabilities can be eliminated by traveling through a *polarization-maintaining single mode optical fiber* but on the cost of increased power instabilities [CKU⁺07]. A ytterbium fiber laser with a coupled collimator⁴ was tested for optical tweezers application. These LASER sources, being single mode fibers are free of pointing instabilities. Power fluctuations did not cause drift during SMFS experiments, in contrast, molecules were observed under constant conditions over thousands of seconds on a regular basis. A LASER stabilization scheme as described in Ref. [CSP09]) is therefore obsolete.

In summary, a number of fixed custom mounts were designed to decrease both, the number of movable parts and the distance the LASER beam travels through the air. The reduced number of components in the optical tweezers' layout and the setup's smaller total footprint improve the stability of the instrument. The use of a piezo nanopositioning mirror tip/tilt actuator⁵ together with the AODs enable independent steering of both traps. This allows for the protein to be kept at a fixed position during SMFS experiments and thus the instrument to be extended by a confocal fluorescence microscope.

4.3.2. Temperature Control

The most significant high-frequency noise source in optical tweezers force spectroscopy experiments is the Brownian motion of the trapped particles. Sample cooling lowers the trapped particles thermal diffusion energy and increases the sample viscosity, thus reduces the noise. Temperature is one of the principal parameters of thermodynamics. In thermodynamical studies, protein un-/folding *pathways* and mechanical stability were found temperature-dependent [LNT⁺10], [JKO⁺03]. Notably, the elastic properties of DNA are temperature dependent [WWRB01]. Measured forces of the B-S-transition of DNA are in good agreement with previous findings [WWRB01].

³ AA OPTO-ELECTRONIC (France): DTSXY-250-1064

⁴ IPG Laser GmbH, (Germany): YLM-5-LP-SC

⁵ Mad City Labs Inc (USA): Nano-MTAX

The setup has been equipped with a water temperature control. A water reservoir is placed ≈ 1 m over the optical setup and connected to collars on the optical tweezers microscope objectives⁶ as well as to the cooling channels of a custom sample holder (figure 77). The flow through the collars and the sample holder is gravity driven. A refrigerated & heating circulator is used to control the reservoir temperature. Sample temperatures of 12–40 °C can be achieved. Sample heating is effective (0.07 K s^{-1}) and no additional noise due to the water flow is observed.

4.3.3. Microfluidic System

A customized microfluidic system⁷ can be used interchangeably with traditional *coverslip sandwich* samples. The custom fluidic chips with $0.17 \mu\text{m}$ thin walls⁸ are fully compatible with the two microscope objectives facing each other. Five laminar flows in the sample chip provide independent measurement environments. The sample holder is a custom design with magnetic fixing points to support non-fluidic samples.

4.3.4. Trapping Potential Calibration

The trapping potential is calibrated prior to each experiment for the specific bead pair used. Thermodynamic diffusion of a trapped bead can be described using Langevin dynamics [BSF04]. Due to sample heating by LASER absorption a calibration of the temperature-dependent viscosity is needed [BSPW⁺06]. A calibration method was used, that overcomes the problem of unknown temperature by measuring the drag coefficient of the bead [TNSH⁺06].

⁶ Biophtechs Objective Cooling Collar – 150303

⁷ Lumicks: μFlux

⁸ Lumicks: custom design

5. Proteins under Mechanical Stress

When in a force spectroscopy experiment force is exerted on a dumbbell, the construct is elongated due to the elasticity of the different polymers. The un-/folding kinetics are force-dependent. Effectively the force exerted on the protein tilts the free energy landscape.

5.1. Polymer Models

The dumbbell end-to-end length—the distance between the beads—and the bead deflection from the trapping center add up to the distance between the trapping centers. A free flexible polymer in solution randomly coils up. This random state of minimal free energy is at maximum entropy. Polymer stretching—as in force spectroscopy experiments—decreases the conformational entropy, causing a restoring force. The *force–extension* relation of unfolded protein chains is well described by the worm-like-chain (WLC) model. A polymer in the WLC model is a continuous and isotropic rod that is bent by thermal energy. The force-dependent mean end-to-end distance of a WLC polymer can be calculated.

A suitable approximation of an unfolded protein in a SMFS experiment uses the protein-specific parameters persistence length p_{prot} and contour length l_{prot} [MS95]. The persistence length is the rod correlation length. Typical values for unfolded proteins are sequence-dependent and similar to the contour length per amino acid 3.65 Å [CCG10]. The contour length corresponds to the completely stretched polymer length. With the thermal energy $k_{\text{B}}T$ the interpolation formula can be written as:

$$F_{\text{WLC}}(x) = \frac{k_{\text{B}}T}{p_{\text{prot}}} \left(\frac{1}{4} \left(1 - \frac{x}{l_{\text{prot}}} \right)^{-2} - \frac{1}{4} + \frac{x}{l_{\text{prot}}} \right) \quad (5.1)$$

The unfolded part of the protein is modeled by a WLC, whereas folded domains are considered inextensible.

The double helical shape of double-stranded DNA causes elastic elongation under force. A modification of the WLC model that accounts for the elastic contribution is called extensible-worm-like-chain (eWLC) model [WYL⁺97]. With the elastic stretch modulus κ the formula reads as:

$$F_{\text{eWLC}}(x) = \frac{k_{\text{B}}T}{p_{\text{DNA}}} \left(\frac{1}{4} \left(1 - \frac{x}{l_{\text{DNA}}} + \frac{F}{\kappa} \right)^{-2} - \frac{1}{4} + \frac{x}{l_{\text{DNA}}} - \frac{F}{\kappa} \right) \quad (5.2)$$

5.2. Model for Force-Dependent Rates in Biological Systems

The first phenomenological descriptions of force-dependent rates in biological systems were observed in a theoretical ligand–receptor unbinding study [Bel78] and force-induced long polymer chain breaking [ZK74]. The model was derived from basic Kramer’s theory [Kra40] and is part of an extended theoretical framework [ER97]. Fluctuations between stable protein conformations are modeled as Brownian diffusion of a Langevin particle in a protein intrinsic one-dimensional double well energy potential $U(x)$.

The external biasing force F in SMFS experiments perturbs the energy potential. This perturbation is approximated by tilting of the potential. Any nonlinear effects are neglected. With the thermal energy $k_B T$, the transition rate from state i to state j is given by:

$$k_{i,j}(F) = k_{i,j}(F = 0) \exp\left(\frac{F \Delta x_i}{k_B T}\right) \quad (5.3)$$

For proline isomerization, the nonlinearities are assumed to be negligible, due to the small change in contour length of around 1 Å. The *Zhurkov–Bell model* (Bell model) is used to describe force-dependent proline isomerization [RMOR14]. If the transition state is close to the initial state, the Bell model performs reasonably well for unfolding. In the scenario of a dumb-bell in an SMFS experiment, the transition state distance corresponds to the whole system, not to the protein alone [ECBM11].

5.3. A Model for Folding in Force Spectroscopy

The linear Bell model does not account for the ideal entropic spring behavior of the unfolded protein chain. The nonlinear contribution in SMFS experiments is considered for in a model of folding against an external force [SBR07]. The model was first developed for *AFM*¹ SMFS experiments and then was adapted for optical trap experiments to account for the nonlinear properties of the elastic DNA linker, of the unfolded protein and the deflection of the beads out of the traps [GBR10]. All energetic contributions of the measurement system are considered in a force-dependent folding model. With the energetic contributions to reach the transition state T from the initial state i to state j , $\Delta G_{i,T}(F_i, F_T)$ the *Berkemeier-Schlierf model* (*B-S model*) can be written as:

$$k_{i,j}(F) = k_{i,j}(F = 0) \exp\left(\frac{\Delta G_{i,T}(F_i = F, F_T)}{k_B T}\right) \quad (5.4)$$

¹ Atomic Force Microscope [BQ86]. The force is exerted on the sample via a soft cantilever. Bending of the cantilever corresponds to the force [RGO⁺97].

6. Force Spectroscopy Protocols

The basic idea of SMFS experiments is to attach some Hookian spring to the sample protein and apply a biasing force. The biasing force acts as a denaturant on the observed protein. Contour length¹ changes due to transitions are observed with high temporal and spatial resolution. The time trajectories obtained during SMFS can be used to unveil full distance resolved energy landscapes of proteins [GBR10]. Different protocols have been developed to determine the energy landscape of kinetic networks of different proteins.

6.1. Passive Mode Force Spectroscopy

Dual-beam optical traps as described in section 4.3 are designed for low drift. Due to their high stability and high detection rates, they are suitable for experiments with constant distance between the trapping centers, exerting a biasing force on the dumbbell. During passive mode experiments, the anchor point of the spring exerting the biasing force on the sample protein is fixed. Typically the biasing force is such that equilibrium fluctuations between different states are observable. The conformational fluctuations cause changes in the bead deflection—providing a force read out.

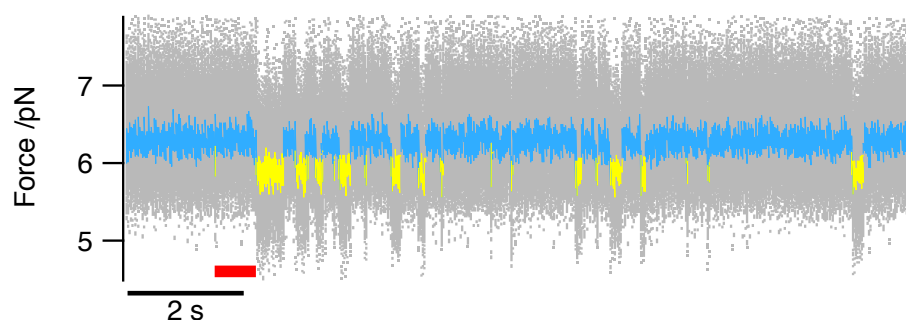


Figure 14 Force time trajectory from a passive mode experiment at a force bias where the folded (smoothed blue) and the unfolded (smoothed yellow) state is populated. The red bar shows the lifetime of the corresponding folded state. Full bandwidth data is shown in gray.

Figure 14 shows a typical time trajectory for a protein that populates the unfolded stretched and its native folded state and thus features two distinct force levels. The unfolded protein chain gets stretched allowing the beads to decrease the deflection, thus exerting a smaller force. The unfolded protein chain is not fully extended (as explained in section 5.1), thus the change in

¹ Contour length denominates the sample protein states end-to-end length at maximum physically possible extension.

deflection is smaller than the contour length change due to the transition. The time between the transition into a state until the transition out, is called its lifetime. The relative cumulative lifetime of a state is called population probability. In general, the folding and unfolding occur under different external biasing forces. When increasing the external force, the most probable protein state shifts from folded to unfolded.

During a typical passive mode SMFS experiment, the biasing force is chosen to observe repeated fluctuations between different states (see figure 14). Unfolded parts of the protein get stretched and increase the distance between the attachment points, thus leading to a drop in the force. Force fluctuations at the anchoring point of the Hookian spring observed during SMFS experiments are directly correlated to the contour length of the protein. The contour length observed during an SMFS experiment is masked with noise. Given good knowledge of the experimental noise, this blur in the passive mode data can be removed by deconvolution and recover the fluctuation's intrinsic probability distribution [GBR10], [WABp⁺06]. For SMFS using optical tweezers, the point spread function can be calculated from first principles [ŽSP⁺13]. The probability distribution of the protein fluctuations $Q_d(x)$ obtained during a long timescale passive mode experiment and its energy landscape $G_d(x)$ are related by the Boltzmann equation:

$$G_d(x) = -k_B T \ln Q_d(x) \quad (6.1)$$

The protein energy landscape reconstruction from passive mode SMFS procedure can be found e.g. in Ref. [RSH⁺14].

6.2. Non-Equilibrium Force Spectroscopy — an Overview

Whenever transition kinetics during equilibrium experiments are slow or individual transition *pathways* are rarely populated, obtaining suitable equilibrium traces gets tedious. Nevertheless, large information content can be accessed by the use of problem-specific non-equilibrium protocols. During non-equilibrium SMFS experiments, the biasing force acting on the sample protein is manipulated to affect transition frequencies or *pathways* in a desired manner. Although it is easier to obtain favored statistics, the interpretation of the data is more complex. Examples of popular non-equilibrium protocols, applicable questions, and analysis methods are presented in the following sections.

6.2.1. The Constant Velocity Protocol

In "constant velocity" SMFS experiments, the traps distance is repeatedly cycled at a constant velocity between a high and a low force. Repeated

stretch-and-relax protocols can be favorable for the investigation of proteins that show a slow folding or unfolding rate. These constant velocity experiments are also used to explore a wide force range in a short time.

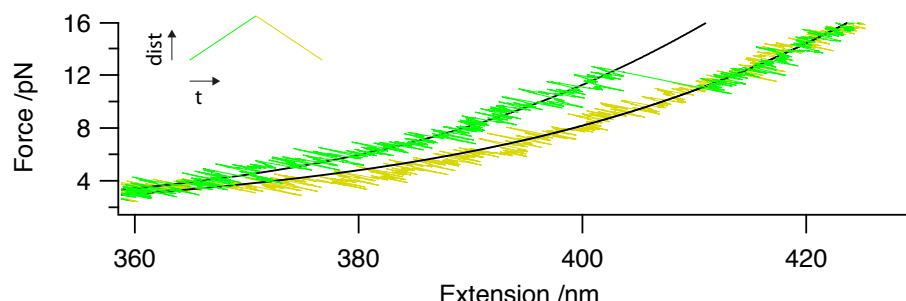


Figure 15 Force-extension diagram from a constant velocity experiment. The stretching (green) with an unfolding event at 12 pN and the relaxation (yellow) of a protein. Shown in black are fits of the WLC and eWLC model (both models are explained in section 5.1). Unfolding length and force are characteristic for a protein. Refolding of the protein is not resolved.

The data obtained during constant velocity experiments are usually displayed in *force–extension* diagrams (figure 15). Proteins show specific un-/folding patterns—so-called fingerprints—in these diagrams. Throughout this work, the specificity of fingerprints during constant velocity experiments were used as quality control. From a number of constant velocity cycles, it is possible to judge whether the dumbbell consists of a single tether and shows typical behavior.

6.2.2. Simple Force Jump Protocols

Kinetic experiments to study dynamics with a high temporal resolution of fast folding proteins often depend on jumps between conditions that induce protein unfolding and folding [EMH⁺00]. In classical double-jump stopped-flow experiments, intermediate states can be unveiled by switching to folding conditions for a short—compared to the folding time of the protein—time span. Back under unfolding conditions, the intermediate states specific unfolding is observed. During similar mechanical SMFS double-jump protocols the folding *pathway* and kinetics of fast folding proteins are observable [SSNR05].

To study protein—protein interaction, often so-called fusion constructs are created. The interaction partners are linked via a flexible inert linker polypeptide. Force jumps have been used to study protein—protein interaction with fusion constructs [GMKD17]. Titin is a large protein in muscles that stretches during muscle elongation, causing a passive restoring force. One anchoring point is titin binding to actinin. A force jump protocol has been used to study the kinetics of the titin actinin complex. Here, force-dependent unbinding was measured at biasing forces which rarely allow the formation of the complex.

6.2.3. Extracting Force-Dependent Unfolding Rates and Energy Landscapes from Non-Equilibrium Experiments

Since non-equilibrium protocols are used to drive transition kinetics and probability distributions, the Boltzmann inversion (equation 6.1) is ineligible to reconstruct the free energy landscape. Un-/folding rates are related to the free energy difference of the different states. Force-dependent transition rates can be obtained from a cycle series that show many transitions [OJO09]. To do so, a constant velocity experiment is described as a series of short passive mode experiments. For a series of identical stretch experiments on a two-state protein, the stretching cycles are divided into force bins. The unfolding rate k_i in bin i is calculated as:

$$k_i = \frac{M_i}{N \cdot \Delta t_i} \quad (6.2)$$

with the number of unfolding events M_i in bin i and the temporal width of the bin Δt_i . Whenever the sample protein is able to fold against force during the relax phase, folding rates can be calculated accordingly. With the forth and back transition rates, the free energy difference of states can be calculated (Appendix B).

Arguably, data from a large number of identical non-equilibrium experiments contain information about the free energy landscape in equilibrium. Fluctuation theorems, e.g. Jarzynski's equality [Jar97] relate free energy difference in equilibrium to an ensemble of finite-time measurements of non-equilibrium work. The work exerted on a protein during a constant velocity stretch or relax cycle corresponds to the area under the *force-extension* diagram (figure 15). The non-equilibrium work distribution of an ensemble of unfolding stretches holds the folding free energy of the protein e.g. [HSK07].

Whenever data on both folding as well as refolding, Crooks' fluctuation theorem provides improved convergence in ensemble size [Cro99]. The full free energy landscape in a two-*pathway* folding scenario has been reconstructed using fluctuation theorems [FLWK15] (for more details see Appendix B).

7. The Fast Force-Jump Protocol

For proteins with complex kinetic networks, not all rates might be easily observable with the methods mentioned above. High-resolution protein folding studies like laser-triggering, temperature jump or fast mixing include prompt changes in conditions to trigger protein folding or unfolding [EMH⁺00]. A comparable force spectroscopy protocol of repeated cycling between two biasing forces can be used to measure the kinetics of weak states that are not detectable in passive mode or constant velocity experiments [SSNR05]. Hence, a fast force jump experiment—in the following referred to as *jump experiment*—protocol was developed to control protein kinetics far out of the equilibrium state. During *jump experiments*, the population of selected states can either be promoted or suppressed. The basic idea of a *jump experiment* is to periodically cycle between force levels that specifically promote the transition from one state to another. During a *jump experiment*, one can set the waiting times (also called *jump dwell times*) at high force τ_h and low force τ_l as well as the force levels themselves within a wide range.

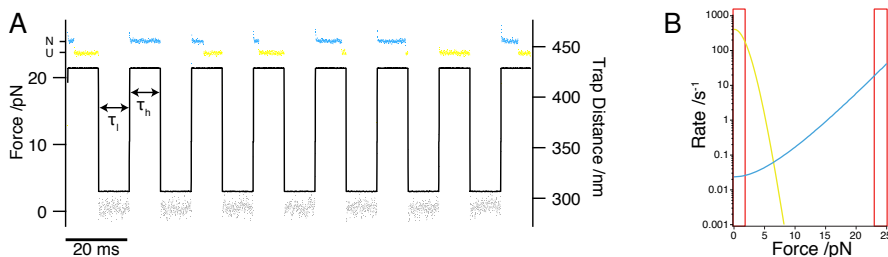


Figure 16 The time trajectory of a typical *jump experiment* is shown in (A). The distance between the trap centers (black, right vertical axis) is switched periodically after jump dwell times at high force τ_h and low force τ_l to alter the force exerting on the protein (red rectangles in B). The force trajectory shows three distinct levels. At F_h , the signal of the transition from the native (blue) to the unfolded (yellow) state is resolved. The signal from folding at low force is too small to be resolved (gray). Colors are assigned by applying the HMM (as explained in section 7.7.2). It is important to note that force changes between values of a few pN and well above 20 pN correlate to a trap distance jump (therefore called force jump), an experimental input. The force fluctuation between the force levels marked with N (for natively folded) and U (unfolded) correlates to a transition event of the protein, an experimental readout. The force-dependent folding (yellow) and unfolding (blue) rates of the protein is described by the *B-S model* (B). At high force only unfolding can occur, at low force refolding is observed exclusively.

During a *jump experiment*, the force changes as a result of a un-/folding transition or a change of the trap distance. Thus steps due to the periodic cycling between different trap distances as well as due to protein unfolding are observed in force trajectories obtained during *jump experiments* (figure 16). In the case of a protein with slow un-/folding rates in equilibrium, *jump experiments* can be used to accelerate the kinetics. It is possible to observe a large number of transitions at a specific biasing force.

Force-dependent protein un-/folding rates as described by the *B-S model* (section 5.3) follow a chevron¹-like exponential behavior with force as the denaturant with a curvature in the low forces regime (figure 16B). Unless noted otherwise, data from *jump experiments* presented in this work was measured using force levels chosen such that folding occurs exclusively at low force and unfolding at high force. During passive mode experiments, the un-/folding occurs under similar forces. This limits the force range suitable for passive mode experiments and might cause a considerable decrease of at least one of the transition rates. To extrapolate folding rates to zero force using the *B-S model* it is important to capture the nonlinear behavior at low force in the experiments, which often is lengthy or hopelessly tedious. During a *jump experiment*, the force is changed rapidly to control the rates individually. In passive mode experiments observed un-/folding rates and population probabilities are coupled. This is visualized by the intersecting curves in the chevron plot (figure 16B). The un-/folding rates during a *jump experiment* can be chosen independently (see the red rectangles in figure 16B). The transition rates between the states are therefore decoupled from the population probabilities. The accessible force range to measure un-/folding rates during *jump experiments* is larger compared to passive mode experiments.

During *jump experiments*, the conformational free energy landscape of a protein is periodically tilted to promote either unfolding or folding (figure 17). A force jump can be used to trigger a protein's transition from one state into another. The ergodic population probabilities of the different states can such be controlled during a *jump experiment*. If a system is perturbed away from its equilibrium state, energy is needed to prevent the system from relaxing back into the equilibrium [Le 84]. This energy is provided by stretching of the dumbbell with the folded inelastic protein and relaxing with the unfolded elastic protein chain (section 5.1), thus causing a shift of the population probabilities.

A hypothetical protein that only populates the native folded and unfolded conformation, is used to discuss the properties of *jump experiments*. During *jump experiments*, the protein is "pushed" repeatedly through its *folding-unfolding-cycle* (figure 17). The back and forth transition is triggered at specific force levels. Thus the transitions occur under differently tilted free energy landscapes. The free energy differences of folding $\Delta G_{U,N}$, and unfolding $\Delta G_{N,U}$ do not add up to zero, resulting in net energy input into the system. Due to the non-constant free energy landscape, the force-dependent un-/folding rates cannot directly be determined from the lifetime of the folded and unfolded state. The apparent folding rate $k_{N,U}^{\text{app}}$ and unfolding rate $k_{U,N}^{\text{app}}$ that are measured by obtaining the lifetimes of the native folded state N and the unfolded protein chain U under changing force are linear combi-

¹ A chevron plot shows how protein folding rates dependent on concentration of denaturant that disrupts the protein's native tertiary structure. It is named after the canonical v, or chevron shape of the log-linear graph.

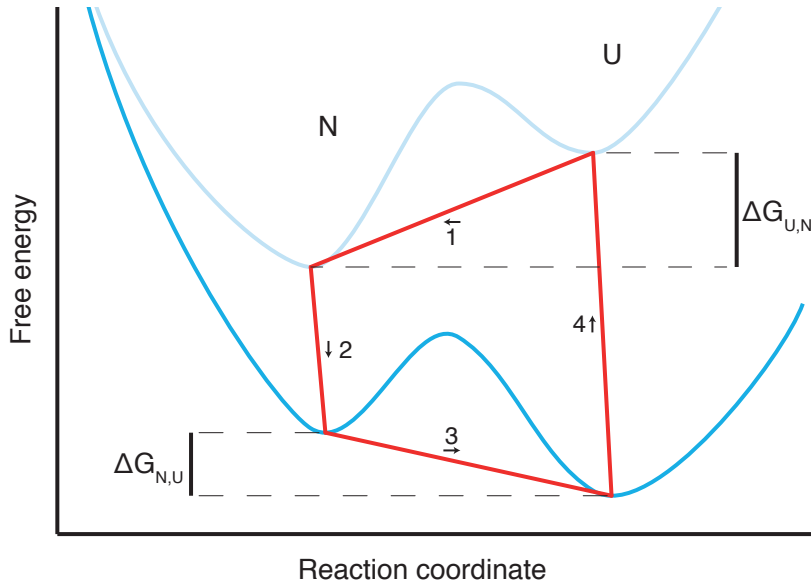


Figure 17 A hypothetical two-state energy landscape at low force (bright blue) and at higher force (dark blue). In a typical *jump experiment*, the forces are chosen such that folding occurs only at low force (1), and unfolding exclusively at high force (3). The energy landscape gets tilted and the protein free energy landscape changes due to the force jump (2 and 4). The free energy difference for folding at low force $\Delta G_{U,N}$ and unfolding at high force $\Delta G_{N,U}$ does not add up to zero. Within every folding-unfolding cycle (1–2–3–4) there is a net energy input into the system.

nations of the force-dependent transition rates $k_{U,N}(F)$ and $k_{N,U}(F)$ at F_h and F_l . With the ergodic probability $p_i(F)$ of being in state i at force level F , the apparent transition rate from state i into state j can be written as:

$$k_{i,j}^{\text{app}} = p_i(F_h)k_{i,j}(F_h) + p_i(F_l)k_{i,j}(F_l) \quad (7.1)$$

To conduct detailed studies on complex kinetic networks, it is necessary to systematically control the population probabilities. In the following sections, models for population probabilities during *jump experiments* are discussed.

7.1. Step Response of the Dumbbell

AODs in optical tweezers instruments for *jump experiments* are chosen for high trap steering speed. The trapping position can be changed within 6.5 μs , using the setup described in section 4.3. The dumbbell (section 4.2) relaxes against the drag of the beads into the new equilibrium position. Only one trap is moved for a force jump, therefore the interpretation of the bead deflection signal is different during the relaxation.

The force signal after a force jump shows overshooting (figure 18). Due to the inertia of the dumbbell and also the DNA handle molecules' elasticity, the force exerted on the sample protein slowly and monotonously transfers

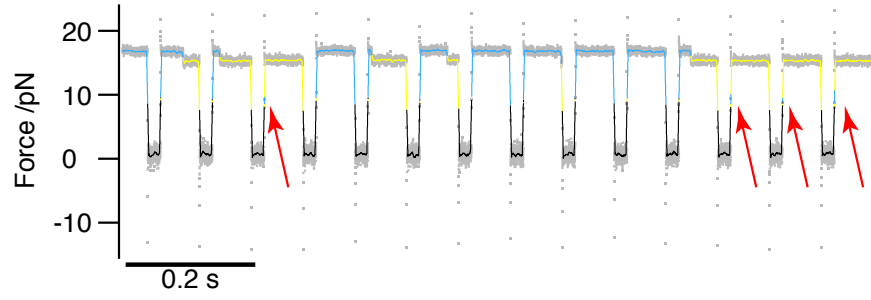


Figure 18 Force trajectory of a two force level *jump experiment*. The force signal shows spikes after the force jumps. For a force jump, the trap position is changed rapidly. Relaxation of the beads into the new trapping position is slower, causing the spikes. The spikes do not correspond to force exerted on the protein. The beads monotonously transition into the new position. This causes misassignment of the state at the force jump position (red arrows).

to the new level. Until the dumbbell has relaxed to its new equilibrium position the signal is corrupted by jump artifacts. The continuous transition of the beads into the new trapping position interferes with Hidden Markov Model algorithm (HMM) state assignment. At force jump positions, false detection of the unfolded state occurs, highlighted by red arrows in figure 18.

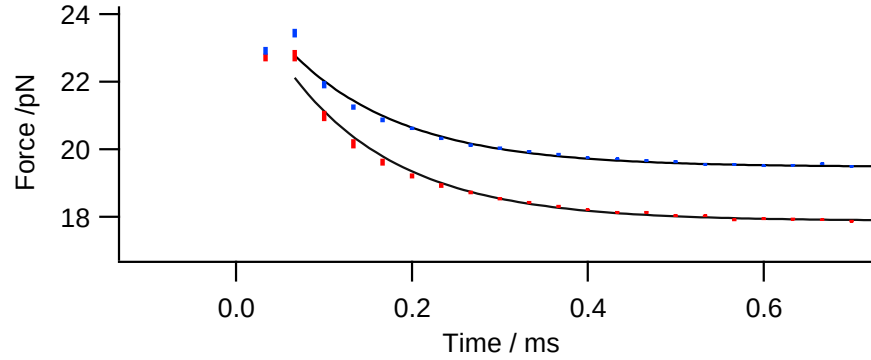


Figure 19 Step response of the force measured after a force jump at 0 s. The force signal of 50 jump events is averaged for each of the native state (blue bars) and the unfolded state (red bars). Fits of single exponential functions to the force signal are shown by the black lines.

Figure 19 shows the averaged force signal of 50 jumps ending up in either the unfolded or the folded state to illustrate the hydrodynamic relaxation. Throughout this work, after each jump, a correction was adopted, where the first 14 data points were substituted by a copy of the following 14 data points (for details see Ref. [GMKD17]).

These 14 data points represent the first 0.5 ms after each force jump. Conformational transitions within that 0.5 ms time window $\tau_0 \leq 0.5$ ms are assigned to the preceding force jump cycle. In typical *jump experiments*, only the high force level is prone to misassignment, due to the low resolution at F_l . The cumulative distribution function $\mathcal{F}_{\tau_0}(t)$ of a transition after jumping to F_h is calculated from f_{τ_0} (equation 7.6), it yields:

$$\mathcal{F}_{\tau_0}(t) = P(\tau_0 \leq t) = \int_0^t f_{\tau_0}(t) = \frac{1 - \exp(-kt)}{1 - \exp(-k\tau_h)} \quad (7.2)$$

The cumulative distribution function at $t = 0.5$ ms gives the fraction of mis-assigned transitions.

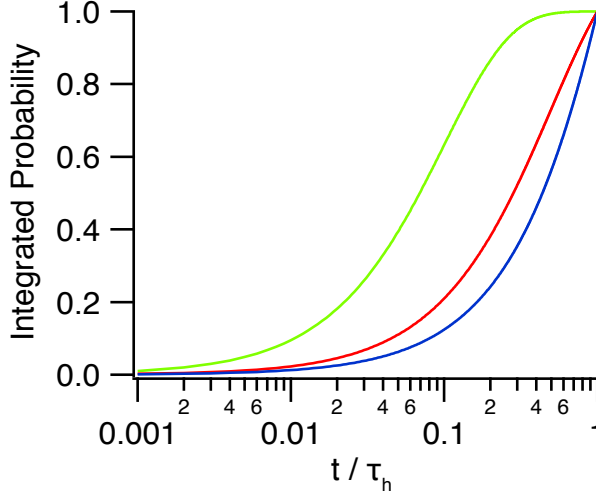


Figure 20 Cumulative distribution functions of τ_0 . Different relative unfolding rates ($k_{N,U} \cdot \tau_h$) are shown: 10 (green), 2 (red) and 0.5 (blue).

For a typical *jump experiment* with an unfolding rate $k_{N,U}(F_h) = 10 \text{ s}^{-1}$ and a jump dwell time at F_h of 20 ms, less than three % of the unfolding events are misassigned. For *jump experiments* with other parameters, the fraction might be higher (see: figure 20).

7.2. The Kinetic Simulation Algorithm

Simulated trajectories are used for forward modeling, or to substantiate theoretical models and analysis methods. Whenever it is too tedious gathering a sufficient amount of data, it might be appropriate to inverse the problem and determine the wanted quantity via simulations. For force spectroscopy experiments, it is a versatile straightforward approach to give the trap distance trajectory and create a full bandwidth state trajectory using *Monte-Carlo* deciders [RSH⁺14]. With suitable contour lengths for the states, as well as, proper protein and DNA parameters, a typical deflection trajectory can be calculated. If noise is added, this time trajectory in many aspects corresponds to the outcome of an SMFS experiment. The full analysis workflow, including state assignment, can be tested on such a simulated trajectory. In a simulation of a *jump experiment*, the frequency bandwidth must well resolve the jump dwell times between jumps and sample a statistically significant number of transition events. Thus full bandwidth

Monte-Carlo simulations are computationally expensive and memory consuming.

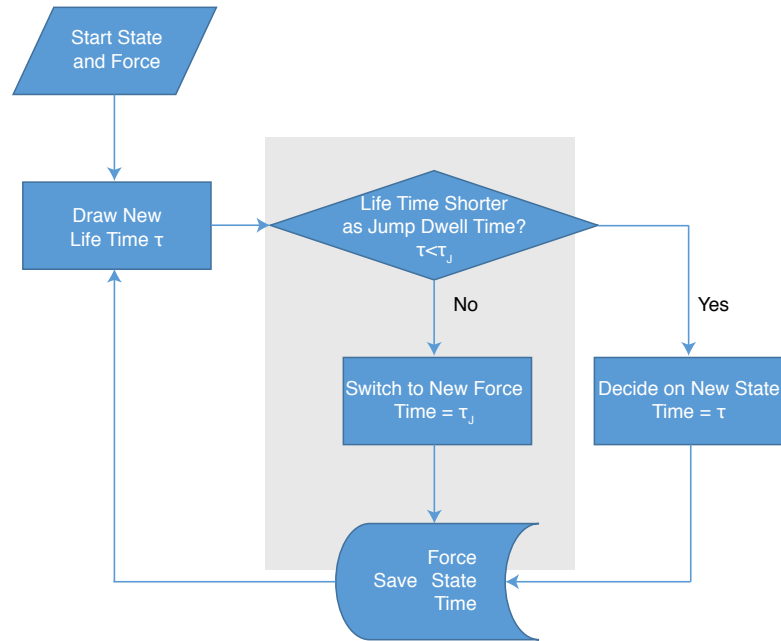


Figure 21 Workflow for simulating a state trajectory based on the Gillespie algorithm. The basic idea is to first create the lifetime of the current state and decide the new state in a second step. If the lifetime exceeds the next force jump τ_j , the state is not changed. A further lifetime after the jump is created. Simulations of *jump experiments* include the additional step for the force jump as shown in the gray box.

Figure 21 shows the workflow for kinetic simulations that efficiently create the time points of transition events, following the Gillespie algorithm [Gil76]. Un-/folding force trajectories during passive mode experiments (as described in 6.1) are created by randomly choosing subsequent dwell times. The mean lifetime of a state s is calculated as $\tau = \frac{1}{\sum_d k_{s,d}}$, where the index d incorporates all states into which s can transition. A lifetime is drawn randomly from an exponential distribution with time constant τ , and the subsequent state is selected according to the branching ratio of the outgoing rates $k_{s,d}$. The periodic event of a force jump is introduced to create un-/folding force trajectories of protein states during *jump experiments*. The lifetimes of subsequent states are drawn according to the Gillespie algorithm until a jump occurs. At the jump event, the time constant for the mean lifetime of the state is changed corresponding to the new force level $\tau(F) = \frac{1}{\sum_d k_{s,d}(F)}$. At the new force level, the Gillespie algorithm starts again (figure 21). With the kinetic simulation, an ensemble of initial dead times and lifetimes for the states and the population probabilities are numerically accessible.

7.3. Quasi-Equilibrium Model of Jump Experiments

Although there is a net energy input to the protein during *jump experiments*, for the quasi-equilibrium model, protein un-/folding transitions are assumed to be spontaneous processes. The probability $p_i(F)$ to populate state $i \in [U, N]$ at force level F , therefore depends only on the ratio of the jump dwell times τ_U and τ_N . With the population probability at a certain force level n , $p_i(F_n) = \frac{\tau_n}{T}$, and T being the period of one jump cycle, the quasi-equilibrium apparent un-/folding rates can be written as:

$$\begin{aligned} k_{U,N}^{\text{app}} &= p_U(F_h)k_{U,N}(F_h) + p_U(F_l)k_{U,N}(F_l) \\ k_{N,U}^{\text{app}} &= p_N(F_h)k_{N,U}(F_h) + p_N(F_l)k_{N,U}(F_l) \end{aligned} \quad (7.3)$$

Jump experiments are designed to study proteins with complex kinetic networks. Kinetics in complex networks often involves a broad range of different rates. For simplicity different jump dwell times were compared to the lifetime of a hypothetical protein's unfolded state during a *jump experiment*.

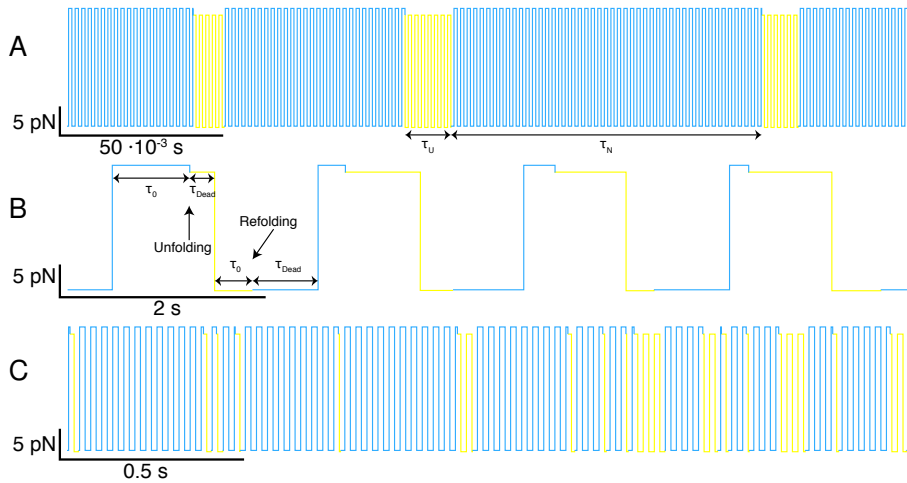


Figure 22 Hypothetical, noise-free un-/folding force trajectories of a protein with two conformational states during a *jump experiment*. Blue curves imply the native state being in effect, yellow curves refer to the unfolded protein chain. The force levels are chosen such, that unfolding exclusively occurs at F_h and folding only at F_l . After unfolding at high force, the protein is not able to fold before the force is switched to F_l . This initial lag is called τ_{Dead} . After folding at F_l there is a similar lag time. The total live time of the folded state τ_N and the unfolded state τ_U are shown in (A). There are 3 different regimes for *jump experiments*: the jump dwell times are short (A), long (B) or similar (C) with respect to the lifetimes of the different states of the observed protein.

There are three different regimes of jump dwell times in a two force level *jump experiment* scenario (figure 22). If the jump dwell times are much smaller than the lifetimes of the states involved in the kinetic network, the protein undergoes multiple jump cycles to F_h and F_l before a folding or unfolding transition occurs (figure 22A). The assumptions of the quasi-equilibrium model are fulfilled, thus the apparent rates are well described by equation 7.3.

In the slow regime, the jump dwell times τ_h, τ_l at the force levels F_h, F_l are chosen such that one conformational transition occurs within every jump dwell time (figure 22B). The force range accessible and the statistics gathered for folding and unfolding transitions are both increased compared to passive mode experiments. After unfolding at high force $\tau_{\text{Dead}}(U_h)$ there is a dead time till the next force jump, likewise there is a τ_{Dead} after folding at F_l . With the mean lifetime of a state i at force level F , being the inverse of the rate $1/k_{i,j}(F)$ and the mean total lifetime of state i , τ_i , the population probabilities of the folded and unfolded state at the different force levels can be written as:

$$\begin{aligned}
 p_U(F_h) &= \frac{\tau_h - k_{N,U}(F_h)^{-1}}{\tau_U} = \frac{\tau_{\text{Dead}}(U_h)}{\tau_{\text{Dead}}(U_h) + \tau_0(U_l)} \\
 p_U(F_l) &= \frac{k_{U,N}(F_l)^{-1}}{\tau_U} = \frac{\tau_0(U_l)}{\tau_{\text{Dead}}(U_h) + \tau_0(U_l)} \\
 p_N(F_h) &= \frac{k_{N,U}(F_h)^{-1}}{\tau_N} = \frac{\tau_{\text{Dead}}(N_l)}{\tau_{\text{Dead}}(N_l) + \tau_0(N_h)} \\
 p_N(F_l) &= \frac{\tau_l - k_{U,N}(F_l)^{-1}}{\tau_N} = \frac{\tau_0(N_h)}{\tau_{\text{Dead}}(N_l) + \tau_0(N_h)}
 \end{aligned} \tag{7.4}$$

If the jump dwell times are long with respect to the lifetimes of the states $\tau_i \gg k_{i,j}^{-1}$, a modified quasi-equilibrium model following equation 7.1 using the probabilities $p_i(F_n)$ of equation 7.4 describes the apparent transition rates. If jump dwell times are similar to the lifetime of one state involved, the situation is more complex (figure 22C). In this regime, the probabilities $p_i(F_n)$ depend on the lifetime of the states $k_{i,j}(F_n)^{-1}$ and the jump dwell times τ_n in a nonlinear manner.

7.4. Step-Resolved Model of Jump Experiments

Optical tweezers are steerable on a timescale of milliseconds (as mentioned in section 4.3 and discussed in more detail in section 7.1). Within the settling time of optical tweezers after a force jump, the force exerted on the protein is undetermined. Data that was recorded within the settling time of the beads (section 7.1), therefore were not analyzed. Thus, there is a practical lower limit for jump dwell times of about 2 ms. During *jump experiments*, using jump dwell times shorter than 5 ms turned out to be experimentally challenging. The time resolution for processes like ligand binding, substrate binding, isomerization etc. is limited by the period of a jump cycle. To measure such processes, it can be necessary to perform *jump experiments* using jump dwell times that are similar to the lifetime of states involved.

In the following sections, general estimators for the lifetime of protein states during a *jump experiment* are derived. The probabilities to be at F_l or F_h for the different states i of the protein $p_i(F_n)$ can be calculated from the

lifetimes. A model is presented, that describes apparent transition rates during *jump experiments* using these probabilities in a un-/folding model for *jump experiments* (equation 7.1).

7.4.1. Initial Dead Time

The lifetime of a protein state consists of two phases, the initial dead time τ_{Dead} and the subsequent lifetime. After a folding or unfolding transition occurred, the rest of the jump dwell is considered as dead time τ_{Dead} (figure 22). The scenarios of folding and unfolding are symmetric, for convenience, only the situation after unfolding is discussed here. To determine an estimator of $E(\tau_{\text{Dead}})$, only jump dwells that show a successful unfolding event and thus a dead time, are relevant. The estimator for the dead time is calculated with τ_h , the total lifetime of the folded state τ_N , the period of a jump cycle T and the time between the jump to F_h until the unfolding event named $\tau_0 = \tau_N \bmod T$ (figure 22):

$$E(\tau_{\text{Dead}}) = \tau_h - E(\tau_0) \quad (7.5)$$

The probability density of unfolding in these dwells depend on $k_{N,U}(F_h)$ and τ_h . To calculate the probability density function $f_{\tau_0}(t)$ all unfolding events is projected into one jump dwell time. For a stochastic unfolding process [Kra40] with a negligible unfolding rate at F_l and the unfolding rate at high force $k = k_{N,U}(F_h)$ the probability density function $f_{\tau_0}(t)$ can be written as:

$$f_{\tau_0}(t) = \begin{cases} 0 & t \notin [0, \tau_h] \\ \sum_{n=0}^{\infty} \exp(-kn\tau_h) k \exp(-kt) = \frac{k \exp(-kt)}{1 - \exp(-k\tau_h)} & t \in [0, \tau_h] \end{cases} \quad (7.6)$$

The probability density (equation 7.6) is only non-zero within one jump dwell time at high force $t \in [0, \tau_h]$.

The estimator of $E(\tau_0)$ is calculated from the probability density function f_{τ_0} (equation 7.6) and can be written as:

$$E(\tau_0) = \int_0^{\infty} t f_{\tau_0}(t) dt = \frac{-k_{N,U}(F_h)\tau_h - 1 + \exp(k_{N,U}(F_h)\tau_h)}{(\exp(k_{N,U}(F_h)\tau_h) - 1) k_{N,U}(F_h)} \quad (7.7)$$

The dead time τ_{Dead} depends on the time τ_0 (equation 7.5) between the unfolding transition and the preceding force jump. If the mean lifetimes of the folded state at high force $k_{N,U}(F_h)^{-1}$ is much shorter than τ_h (green line in figure 23), τ_0 equals the lifetime of the folded state:

$$\lim_{k_{N,U}(F_h) \rightarrow \infty} E(\tau_{\text{Dead}}) = \tau_h - \frac{1}{k_{N,U}(F_h)}$$

The opposite limit of short jump dwell time at high force τ_h and long lifetimes of the folded state, the probability density function is flat between 0 and τ_h

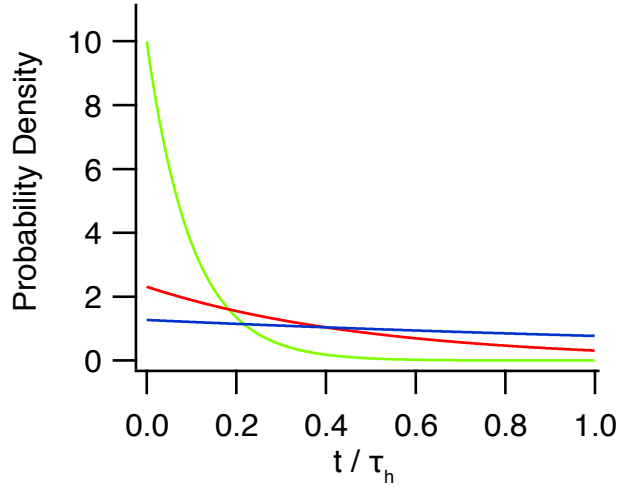


Figure 23 Unfolding probability density functions for a dwell with an unfolding event. Different relative unfolding rates ($k_{N,U} \cdot \tau_h$) are shown: 10 (green), 2 (red) and 0,5 (blue).

(blue line in figure 23). The dead time in this scenario is:

$$\lim_{k_{N,U}(F_h) \rightarrow 0} E(\tau_{\text{Dead}}) = \frac{\tau_h}{2}$$

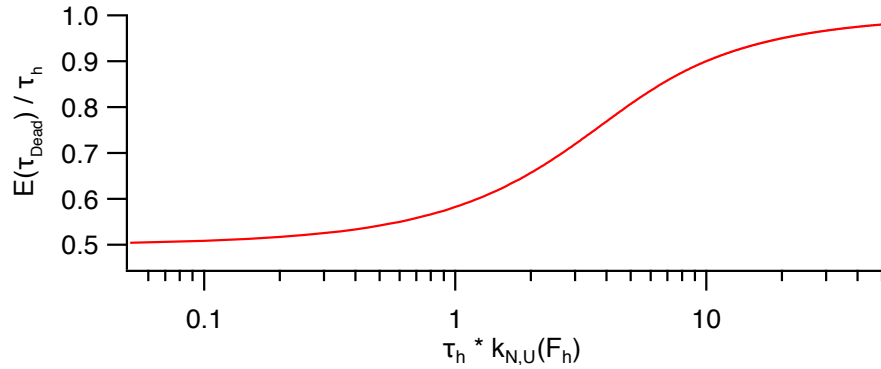


Figure 24 Initial dead time after protein unfolding. For slow unfolding kinetics, with respect to τ_h unfolding appears random. The mean dead time, therefore, is $\frac{1}{2}\tau_h$. For fast unfolding the mean dead time becomes $\tau_h - \tau_N(F_h)$.

The dead time τ_{Dead} depends on the time τ_0 between the unfolding transition and the preceding force jump. Figure 24 shows how the mean relative dead time $E(\tau_{\text{Dead}}) \cdot \tau_h^{-1}$ depends on the ratio of jump dwell time at high force and the total lifetime of the native state $\frac{\tau_h}{\tau_N}$, or with respect to the unfolding rate $\tau_h \cdot k_{N,U}(F_h)$.

7.4.2. Life Times of Protein States during Jump Experiments

To calculate a mean protein state lifetime during a *jump experiment* the time dependency of the folding rate $k_{U,N}(F(t)) = k_{U,N}(t)$ is explicitly accounted for. In the following section the estimator of protein state lifetimes is calculated:

$$E(\tau_U) = \int_0^{\infty} t f_{\tau_U}(t) dt \quad (7.8)$$

After the initial dead time (section 7.4.1) the force bias on the protein periodically changes back and forth between F_h , at which refolding is highly improbable, and F_l to allow refolding. After the initial dead time, the folding rate frequently interchanges between two values, due to force jumps. The probability density function of folding, with times at low force l :

$$l = \{[\tau_{\text{Dead}} + n(\tau_h + \tau_l), \tau_{\text{Dead}} + n(\tau_h + \tau_l) + \tau_l] | n \in \mathbb{N}_0\} \quad (7.9)$$

can be written as:

$$f_{\tau_U}(t) = \begin{cases} k_{U,N}(F_h) \cdot \exp(-k_{U,N}(F_h) \cdot t) & t \notin l \\ k_{U,N}(F_l) \cdot \exp(-k_{U,N}(F_l) \cdot t) & t \in l \end{cases} \quad (7.10)$$

The characteristics of the lifetime distributions during *jump experiments* are visualized in a cumulative distribution² plot. The cumulative distribution function $P(\tau_U < t) = \int_0^t \tau \cdot f_{\tau_U}(\tau) d\tau$ —the probability that the protein already folded—for a passive mode experiment is exponential.

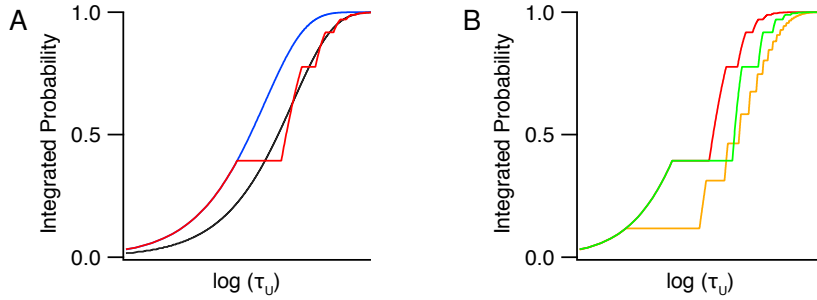


Figure 25 Hypothetical integrated lifetime histograms of the unfolded state for different *jump experiment* scenarios. The folding rate at F_h is zero. (A) Symmetric *jump experiments*, featuring identical jump dwell times τ_h and τ_l . Blue line: $\tau_l \gg k_{U,N}(F_l)^{-1}$, folding will occur within one phase at F_l and the lifetime distribution is exponential with an estimated lifetime of $k_{U,N}(F_l)^{-1}$. If $\tau_l \ll k_{U,N}(F_l)^{-1}$, folding will occur after many *force-jump-cycles* exerting F_h on the protein, thus preventing folding for half of the time. The estimated lifetime is $2 \cdot k_{U,N}(F_l)^{-1}$ (black line). For $\tau_l \approx k_{U,N}(F_l)^{-1}$, folding occurs within a couple of *force-jump-cycles*. The lifetime distribution features pronounced steps and is not a single exponential (red line). (B) comparison of the red scenario in (A), with longer τ_h , as depicted by the green line, and shorter τ_l shown in yellow.

² Continuous equivalent of a cumulative histogram. It gives the cumulative fraction of observations in a sample up to the specified time.

For *jump experiments*, the unfolded state's cumulative lifetime distribution function $P(\tau_U < t)$ shows steps of flat and steep phases (as shown in figure 25). These steps separate the different phases during which F_h or F_l is applied with the optical tweezers. The limit for slow *jump experiments* is a passive mode experiment in which all observed unfolding events occur within the first dwell (blue line in figure 25A). In the limit of negligible jump dwell time the steps vanish since there is not more than one event per step, and the integrated lifetime histogram shows single exponential lifetimes distribution, with an apparent rate $\frac{1}{2}k_{U,N}(F_l)$ (black line in figure 25A). In cumulative histograms of unfolded lifetimes, steps are particularly prominent for $\tau_l \approx k_{U,N}(F_l)^{-1}$ (red line in figure 25A). To calculate the estimator of the unfolded lifetime, the integral in equation 7.8 is split into sections of a jump dwell time τ_{dt} . In a first step for $F = F_l = F_h$, the section-defined integral (equation 7.8) can be re-written as:

$$E(\tau_U) = \sum_{n=0}^{\infty} \int_0^{\tau_{dt}} \underbrace{(\exp(-k_{U,N}\tau_{dt}))^n}_{P(t < n\tau_{dt})} \cdot \underbrace{k_{U,N}(n\tau + t) \exp(-k_{U,N}t)}_{kt \exp(-kt)} dt \quad (7.11)$$

An explicit expression for the lifetime of a protein's state during two force level *jump experiments* is derived in the following. The times of the splitting points are given by the sum of all previous jump cycles and the dead time τ_{Dead} (equation 7.7):

$$\begin{aligned} t_l^n &= \tau_{Dead} + n(\tau_h + \tau_l) \\ t_h^n &= \tau_{Dead} + \tau_l + n(\tau_h + \tau_l) \end{aligned} \quad (7.12)$$

In the following, non-negligible folding rates at high force $k_{U,N}(F_h)$ are taken into account. To calculate the mean lifetime, the cumulative distribution function of the lifetime $P(\tau_U < t) = \int_0^t f_{\tau}(t) dt$ at the splitting points are pre-calculated:

$$\begin{aligned} P(\tau_U < \tau_{Dead}) &= \exp(-k_{U,N}(F_l)\tau_{Dead}) \\ P(\tau_U < t_h^n) &= P(\tau_U < \tau_{Dead}) \left(\exp(-k_{U,N}(F_h)) \exp(-k_{U,N}(F_l)) \right)^n \\ P(\tau_U < t_l^n) &= P(\tau_U < \tau_{Dead}) \left(\exp(-k_{U,N}(F_h)) \right)^n \left(\exp(-k_{U,N}(F_l)) \right)^{n+1} \end{aligned} \quad (7.13)$$

The estimator of the lifetime is calculated by adding up the finite integrals of the probability densities multiplied by the time. The estimators for the unfolded lifetime at low force $E(\tau_U^l)$ and high force $E(\tau_U^h)$ yield:

$$\begin{aligned}
E(\tau_U^h) &= \int_0^{\tau_{\text{Dead}}} k_{U,N}(F_h)t \cdot \exp(-k_{U,N}(F_h)t) \\
&\quad + \sum_{n=0}^{\infty} \int_0^{\tau_h} P(\tau_U < t_h^n) \cdot (t + t_h^n) k_{U,N}(F_h) \cdot \exp(-k_{U,N}(F_h)t) dt \quad (7.14) \\
E(\tau_U^l) &= \sum_{n=0}^{\infty} \int_0^{\tau_l} P(\tau_U < t_l^n) \cdot (t + t_l^n) k_{U,N}(F_l) \cdot \exp(-k_{U,N}(F_l)t) dt
\end{aligned}$$

The estimator for the lifetime of the unfolded state (equation 7.8) is obtained via:

$$E(\tau_U) = E(\tau_U^h) + E(\tau_U^l) \quad (7.15)$$

The folded state's lifetime estimator is calculated in an analogous manner.

7.4.3. Force-Dependent Population Probabilities of Protein States during *Jump Experiments*

For protein interactions with other proteins, ligands, substrates or enzymes the probabilities to be at F_l or F_h for the different protein states i , $p_i(F_n)$ is crucial. The initial dead time after a folding or unfolding transition affects the force-dependent population probability $p_i(F_n)$. To determine the population probabilities the force-dependent mean lifetimes of the protein states need to be known. With the folded state's lifetime estimator $E_h(\tau_N)$ for a protein that folded at high force and $E_l(\tau_N)$ for low force folding, together with the probability for folding at high force $p_{U,N}^h$ and at low force $p_{U,N}^l$ the total lifetime estimator of the unfolded state is given by:

$$E(\tau_N) = p_{U,N}^l \cdot E_l(\tau_N) + (1 - p_{U,N}^l) \cdot E_h(\tau_N) \quad (7.16)$$

In this first step, the negligibility of folding at F_h is checked. For a protein that is able to fold during *jump experiments*, $p_{U,N}^h$ and $p_{U,N}^l$ add up to one. The probability for folding at either F_h or F_l is calculated with equation 7.11:

$$p_{U,N}^h + p_{U,N}^l = \int_0^{\infty} f_{\tau_U}(t) dt = 1 \quad (7.17)$$

The probability density function of folding at low force $f_{\tau_U}^l(t)$, with times at low force l (equation 7.9) can be written as:

$$f_{\tau_U}^l(t) = \begin{cases} 0 & t \notin l \\ k_{U,N}(F_l) \cdot \exp(-k_{U,N}(F_l) \cdot t) & t \in l \end{cases} \quad (7.18)$$

The refolding probability at low force can be calculated by integrating the probability density function over the times being unfolded at low force (equation 7.18). The probability for folding at F_l after unfolding at F_h yields:

$$p_{U,N}^l = \sum_{n=0}^{\infty} \int_0^{\tau_n} P(\tau_U < t_l^n) \cdot k_{U,N}(F_l) \cdot \exp(-k_{U,N}(F_l)t) dt \quad (7.19)$$

The refolding probability at high force depends on the jump dwell times and the difference of the folding rates $k_{U,N}(F)$ at the two force levels. In a typical *jump experiment*, the jump dwell times τ_l and τ_h are within one order of magnitude. Whereas the folding rates at the different force levels differ by multiple orders of magnitude to drive the folding-unfolding kinetics.

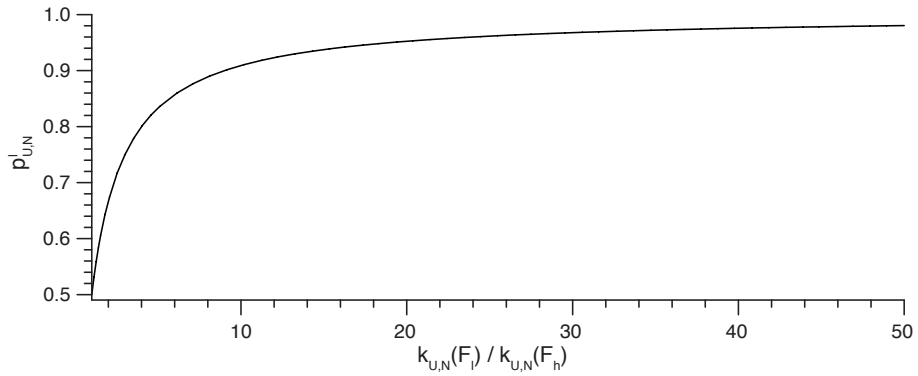


Figure 26 The probability that refolding occurs at low force $p_{U,N}^l$ equation 7.19 during a *jump experiment* with both jump dwell times 10^{-2} s and the folding rate at high force $k_{U,N}(F_h)$ fixed at 10^{-3} s^{-1} .

Thus, the probability to fold at F_l during a 'symmetric' *jump experiment* with jump dwell times of 10 ms, as shown in figure 26 is checked for negligible folding at F_h . During most *jump experiments* the folding rate at F_l is at least 100 times faster compared to the folding rate under F_h . For such *jump experiments*, it is a good approximation, to assume that folding appears only at low force. Lifetimes of the folded state τ_N can be calculated accordingly, leading to the approximation that unfolding occurs at F_l exclusively.

With the estimators of the lifetimes at the different force levels (equation 7.14), population probabilities for the unfolded state in a two force level *jump experiment* p_U^h and p_U^l are given by:

$$p_U^h = \frac{E(\tau_U^h)}{E(\tau_U) + E(\tau_N)} \quad (7.20)$$

$$p_U^l = \frac{E(\tau_U^l)}{E(\tau_U) + E(\tau_N)}$$

In the scenario presented here, the four states of interest — native at high force N_h , native at low force N_l , unfolded at high force U_h and unfolded at

low force U_l —are populated with a probability depending on jump dwell times as shown in figure 27.

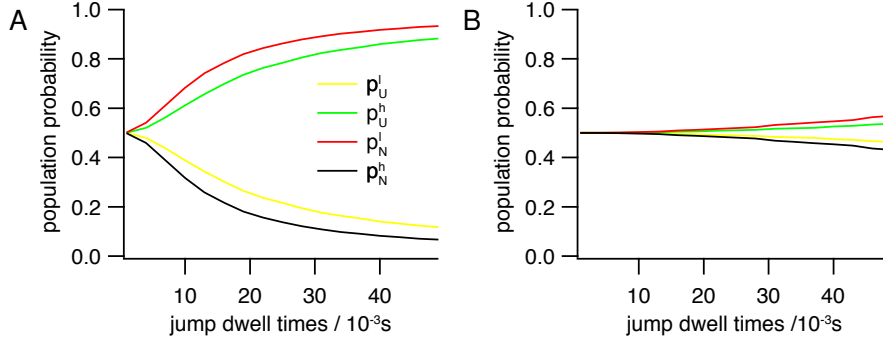


Figure 27 Hypothetical population probabilities of the protein states at the force levels during a *jump experiment* applying equal dwell times for both force levels. The unfolding rate at low force $k_{N,U}(F_l)$ and refolding at high force $k_{U,N}(F_h)$ are zero. The refolding rate at low force $k_{U,N}(F_l)$ is 100 s^{-1} in (A) and 10 s^{-1} in (B), the unfolding rate at high force $k_{N,U}(F_h)$ is 200 s^{-1} in (A) and 20 s^{-1} in (B). The population probability of the unfolded state at high force p_N^h in black, at low force p_N^l in red and of the unfolded state at high force p_U^h in green, at low force p_U^l in yellow.

7.4.4. Summary

In this part, a *jump experiment* protocol for SMFS using optical tweezers was introduced. Theoretical considerations resulted in two models that describe *jump experiment* properties. A simple, quasi-equilibrium model for *jump experiments* (section 7.3) predicts population probabilities for constant dwell time ratios $\frac{\tau_l}{\tau_h}$ independent of the value of the jump dwell times. Population probabilities of the protein states depend only on the ratio of the jump dwell times and the un-/folding kinetics at the different force levels. Explicit consideration of the protein's time-dependent free energy landscape unveils nonlinear population probability dependence from *jump experiment* parameters, e.g. the value of the jump dwell times (section 7.4). Shown in figure 27, the quasi-equilibrium approximation is useful only for lifetimes of the states k^{-1} that are much longer compared to the jump dwell times τ_l and τ_h . Experimental limitations often set a lower limit for the jump dwell times. Many experiments will exceed the limits of the quasi-equilibrium model, thus data analysis presupposes precise experimental determination of the kinetic rates and population probabilities.

7.5. Extracting Zero Force Folding Rates from *Jump Experiments*

Jump experiments drive protein kinetics far out of equilibrium. While the unfolding kinetics are often directly observable, the contour length change due to folding is not resolved at the low force level most of the times. If the contour length of the native state is unique, the folding kinetics can be

observed in force jump experiments [SSNR05]. Folding of cis-FLNa20 is observable by choosing the high force level such that the native trans-state is too short-lived to be detected. Short unfolded lifetimes—comparable to the jump dwell times—are not Markovian. Folding is triggered by a jump to low force, thus the rate analysis (as explained in section 7.7.3) is not suitable for such fast folding transitions. The following definitions are used to derive a suitable likelihood function of the folding rate at low force in such a scenario. The survival function of the unfolded state with the folding rate k is given by:

$$S_k(t) = \exp(-kt) \quad (7.21)$$

The survival function $S_k(t)$ represents the probability to still find the unfolded state after a waiting time t . The opposite view, the probability that the protein is already folded after a period t is given by the cumulative hazard function:

$$\Lambda_k(t) = 1 - \exp(-kt) \quad (7.22)$$

In this scenario, it is observed whether folding of the protein occurred within the first dwell time at low force, or later. Thus a variable δ is defined to indicate censored data, after one dwell at low force $t = \tau$:

$$\delta_n = \begin{cases} 1 & t_n > \tau_h \\ 0 & t_n < \tau_h \end{cases} \quad (7.23)$$

The outcome of typical *jump experiment* is a number n_{tot} of observed folding times t_n . The lifetimes are observed at high force, thus the lifetime is compared with the jump dwell time at the corresponding force level τ_h . The likelihood function $L(k, t)$ discriminates between folding in the first attempt and later ones.

$$L(k, t) = \prod_{n=0}^{n_{\text{tot}}} S_k(\tau)^{\delta_n} \Lambda_k(\tau)^{1-\delta_n} \quad (7.24)$$

The maximum of $L(k, t)$ is the maximum likelihood estimator (*MLE*) for the folding rate $k_{U,N}$ and is given by:

$$E_{\text{MLE}}(k_{U,N}(F_l)) = -\frac{\ln(\frac{\sum_n \delta_n}{n_{\text{tot}}})}{\tau_l} \quad (7.25)$$

If all observed dwell times are shorter than τ_h the *MLE* for the folding rate is infinite, if all are longer the *MLE* is zero. Thus, appropriate *jump experiment* parameters are chosen such that dwell times shorter, as well as longer than τ_h are observed. Error analysis based on bootstrap confidence intervals analog to section 7.7.3 is suitable.

7.6. Proline Isomerization-Dependent Folding During *Jump Experiments*

In some proteins, proline isomerization engenders two distinct native states with different un-/folding kinetics. The force-induced *cis-trans*-isomerization of proline bond p2225 in human filamin A, significantly increases the binding affinity for transmembrane proteins, thus acting as a force sensor [RMOR14].

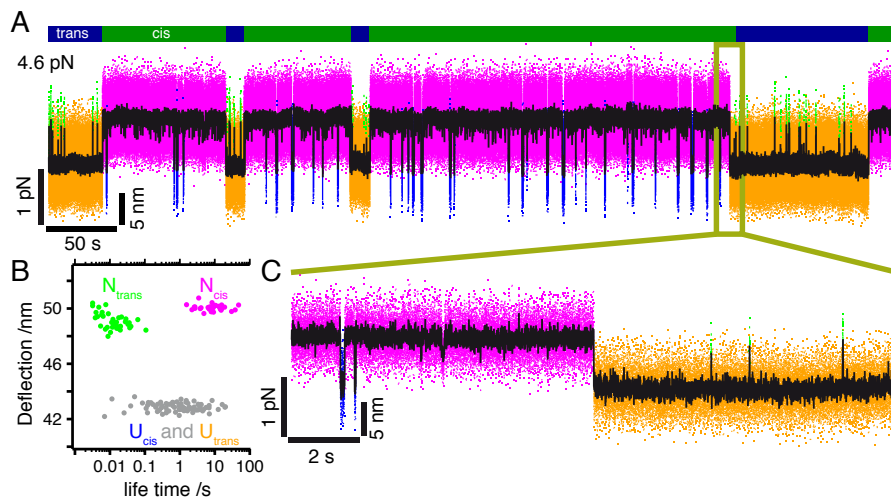


Figure 28 Force-dependent equilibrium folding kinetics of FLNa20. (A) shows a FLNa20 un-/folding force trajectory obtained during a passive mode experiment under a biasing force of 4.6 pN (exerted on the unfolded protein chain). The colored trajectory corresponds to 5 kHz data where the stable long-lived native state N_c is colored in pink, the less stable short-lived N_t in green, the short-lived unfolded U_c in blue and the long-lived unfolded U_t orange, smoothed data in black. (A, Upper) The horizontal bar denotes the cis (in green) and trans (in blue) states. The expand (C) shows a *cis-trans*-isomerization and illustrates the different stability of the folded states. (B) A deflection over lifetime plot shows that the two native states N_c and N_t are kinetically well separated. Adapted from [RMOR14].

The isomerization state of p2225, within FLNa20, controls the native state folding free energy difference. FLNa20 populates four distinct states, U_t , U_c , N_t , and N_c . The different folding kinetics provide a possible read out of the isomerization state during passive mode SMFS experiments. Figure 28 shows an example of such an experiment. The trap distance is chosen such that hopping between the native and unfolded is observable for both isomerization states. The trans and cis-states are mapped on the un-/folding force trajectories, via the un-/folding kinetics. The temporal resolution is limited to the un-/folding kinetics. The trans and the cis native states are clearly separated in the bead deflection over lifetime scatter plot (figure 28B). Unfolded states flanking N_t are assigned U_t . Since the isomerization kinetic is much slower than the un-/folding kinetics, it is possible to measure the lifetimes of the cis and trans-states and calculate proline isomerization rates.

Proline isomerization is catalyzed by a class of enzymes therefore called

PPlase. In the force regime that allows the observation of un-/folding fluctuations of cis and trans-FLNa20, un-/folding transitions and enzymatic proline isomerization are not distinguishable. To study the catalytic mechanism of PPlases, *jump experiments* as explained in chapter 7 are suitable.

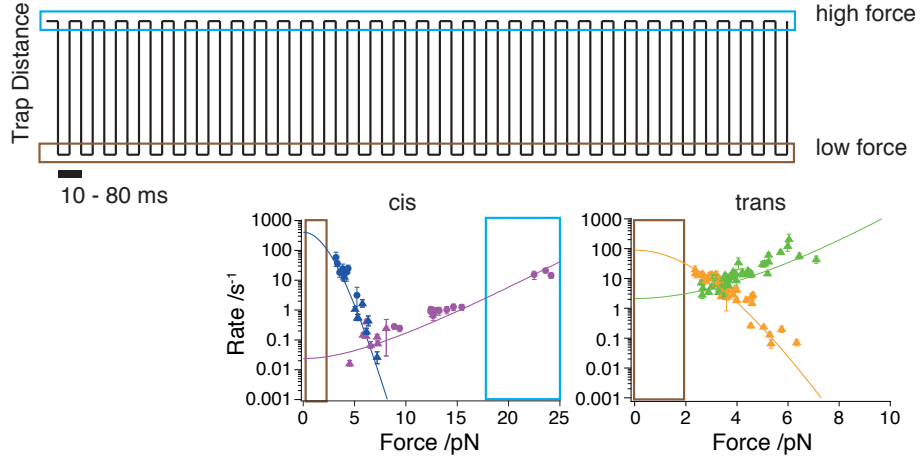


Figure 29 Basic idea of *jump experiments*. The jump dwell times are chosen short enough to resolve the lifetimes of cis and trans-states in presence of PPlases. The un-/folding transitions are only resolved at high force F_h (blue rectangle in the top part). The high force level is chosen such that the force-dependent unfolding rate from N_t is too fast to be resolved and the N_t lifetime is well resolved (bottom panel). With that, every observed folded state corresponds to N_c .

The un-/folding kinetics can be considerably increased, due to the independent force levels for un-/folding during a *jump experiment* (figure 29). The accelerated un-/folding kinetics facilitate the temporal resolution of enzymatic proline isomerization. Folding rate analysis procedures of such complex networks are explained in detail in section 7.5. In FLNa20, the long-lived unfolded state (orange in e.g. figure 28) corresponds to p2225 trans phases.

The un-/folding lifetimes are limiting the temporal resolution. Under forces, that show reasonable short N_c lifetimes, N_t is undetectable short-lived. Phases with consecutive unfolding events (figure 30 red arrows) are assigned to the cis-state (green in figure 30). In a force over lifetime plot (figure 48B), a threshold on the unfolded lifetimes is used to assign trans phases (figure 30 marked in dark blue). If the cis and trans-states were assigned to the high force trace after post-processing (section 7.7.1), cis and trans phases are multiplied by the jump dwell time ratio $t_n = t_n^h \frac{\sum_j \tau_j}{\tau_h}$ to calculate the corresponding lifetimes. The rate out of an isomerization state — $k_{c,t}^{\text{app}}$ and $k_{t,c}^{\text{app}}$ (equation 7.26) — can be calculated from the cis and trans lifetimes t_n using equation 7.34. Apparent isomerization rates obtained during *jump experiments* are a superposition of different possible transitions. The apparent rates are composed of different components:

$$\begin{aligned} k_{c,t}^{\text{app}} &= p_{N_c} k_{c,t}^N + p_{U_c}^l k_{c,t}^U(F_l) + p_{U_c}^h k_{c,t}^U(F_h) \\ k_{t,c}^{\text{app}} &= p_{N_t} k_{t,c}^N + p_{U_t}^l k_{t,c}^U(F_l) + p_{U_t}^h k_{t,c}^U(F_h) \end{aligned} \quad (7.26)$$

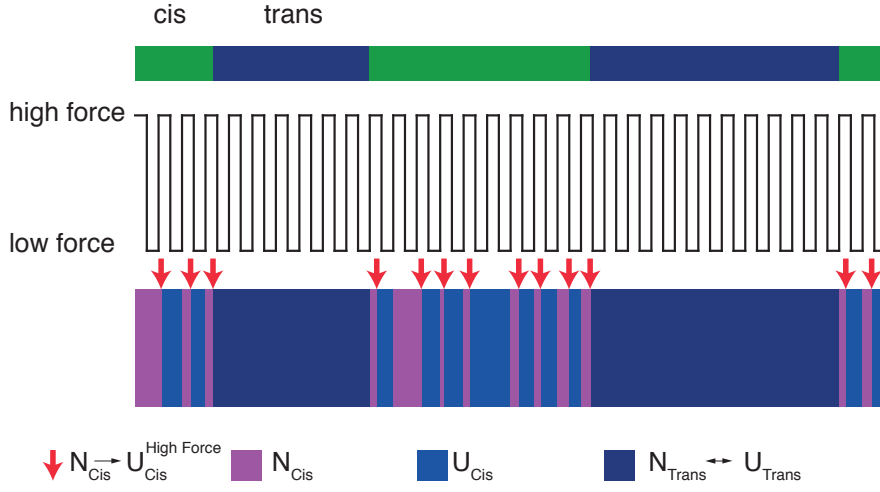


Figure 30 In data obtained during *jump experiments*, three states are resolved. The black line indicates the traps' distance. Forces are chosen such that observed folded states are N_c (pink) exclusively. The N_c unfolding events are highlighted with red arrows. Short-lived (in bright blue) and long-lived (in dark blue) unfolded states are distinguishable. The long-lived unfolded states are assigned trans (top blue bars). Phases of fast un-/folding kinetics are assigned cis (top green bars).

During *jump experiments*, cis and trans lifetimes t_c and t_{trans} , as well as, the time spent in the unfolded states U_c and U_t at high force $t(U_c^h)$ and $t(U_t^h)$ can be observed.

Native state isomerization is assumed force independent. P2225 is part of a loop buried in the structure of the FLNa20 construct thus shielded from forces in the folded state. The population probabilities p_j^F of a state j at force F are sensitive to the experimental parameters (details: section 7.4.3) and therefore need to be determined from the data. Force-dependent pro-line isomerization from state s_i to state s_j with $s \in [U, N]$ and $i, j \in [c, t]$ is described by the Bell model (section 5.2):

$$k_{s_i, s_j}(F) = k_{s_i, s_j}(F = 0) \cdot \exp\left(\frac{F \Delta x}{k_B T}\right) \quad (7.27)$$

The following procedure is specific to FLNa20 and two force level *jump experiments* with parameters chosen as in figure 29. The cis and trans dwell times with $i \in [U, N]$ and $F \in [F_l, F_h]$ are directly assigned to the data (figure 30).

$$\begin{aligned} t_{cis} &= \sum_{F, i} t(i_c^F) \\ t_{trans} &= \sum_{F, i} t(i_t^F) \end{aligned} \quad (7.28)$$

The population probabilities of U_c are calculated from the corresponding lifetimes $t(U_c^F)$ and the total cis lifetime. At high force, the lifetimes of U_c are assigned via HMM. At low force, there is no signal corresponding to

folding events. Thus, the *MLE* of the folding rate (equation 7.25) is used to estimate the cumulative lifetimes.

$$t(U_c^l) = \frac{n_{\text{tot}}}{E_{\text{mle}}(k_{U,N}(F_l))} \quad (7.29)$$

From the times spend in each state the population probabilities for cis-FLNa20 are given by:

$$\begin{aligned} p_{U_c}^F &= \frac{t(U_c^F)}{t_c} \\ p_{N_c}^F &= 1 - \frac{\sum_F t(U_c^F)}{t_c} \end{aligned} \quad (7.30)$$

The experimental protocol to measure enzymatic proline isomerization is based on negligible N_t lifetimes at high force $p_{N_t}^h \rightarrow 0$. The N_t state exclusively lives at low force, thus a τ_h independent apparent low force proline isomerization rate can be defined:

$$k_{t,c}^{\text{app},l}(F_l, \tau_l) = (p_{N_t} k_{t,c}^N + p_{U_t}^l k_{t,c}^U(F_l)) \frac{\tau_h + \tau_l}{\tau_l} \quad (7.31)$$

The jump dwell times at the different force levels relate to the population probabilities:

$$\begin{aligned} p_{U_t}^h &= \frac{\tau_h}{\tau_h + \tau_l} \\ p_{N_t} + p_{U_t}^l &= \frac{\tau_l}{\tau_h + \tau_l} \end{aligned} \quad (7.32)$$

For trans-FLNa20, the high force level is exclusively populated by U_t . The apparent isomerization rate (equation 7.26) then yields:

$$k_{t,c}^{\text{app}} = (p_{N_t} + p_{U_t}^l) k_{t,c}^{\text{app},l}(F_l, \tau_l) + p_{U_t}^h k_{t,c}^U(F_h) \quad (7.33)$$

The isomerization rates $k_{c,t}^N$, $k_{t,c}^N$, $k_{t,c}^U(F)$ and $k_{c,t}^U(F)$ need to be extracted from a series of different *jump experiments*.

7.7. Analysis of Un-/Folding Force Trajectories obtained during *Jump Experiments*

By using the *fast-force-jump* protocol, certain experimental challenges can be overcome and otherwise hidden details revealed. In data obtained during *jump experiments* only a part of the transitions involved are directly observable and typically there is no signal at low force. Bead deflection—proportional to the biasing force—is used as a signal for analysis. For the different force levels during a *jump experiment*, the interpretation of bead deflection in terms of the conformational state is heterogeneous. Furthermore, a *jump experiment* force trajectory shows artifacts due to finite dumb-

bell step response. Prior to data analysis, the data from the individual force levels are separated and force jump specific corrections are applied. In a second step, the protein state trajectory is extracted for further analysis.

7.7.1. Preparation of Data obtained during *Jump Experiments*

Force-dependent lifetime analysis of data observed during *jump experiments* is performed for the different trap distance levels individually. Data collected at the same trap distance are merged to form a *passive-mode-like* trajectory, enabling an HMM-based analysis. *Jump experiments* can include more than two trap distance levels, the data preparation is performed independently of the trap distance level count.

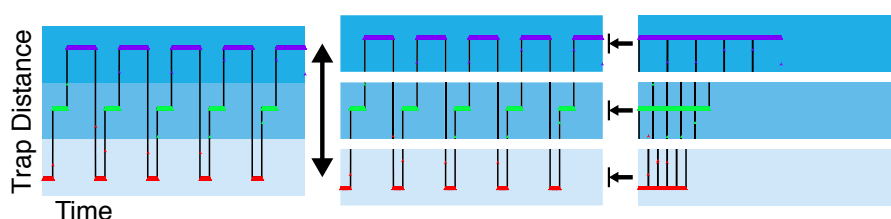


Figure 31 A cartoon representation of post-processing data obtained in a typical *jump experiment*, is visualized for an experiment with three trap distance levels. The distinct force levels are separated and the trunks of data are merged to continuous un-/folding force trajectories.

Figure 31 shows the data preparation procedure for the scenario of a three force level *jump experiment*. The low force level provides fast folding, the mid force level probes the folded states and the high force level ensures unfolding (detailed example in chapter 11). Before the un-/folding force trajectory is further analyzed, each point is categorized according to the corresponding trap distance. For force level classification a *k-means clustering* algorithm is used on the trap distance signal [Llo82]. The data from each trap distance are merged into a continuous trajectory (figure 31). To automate the unfolding rate calculation on these time trajectories, HMM analysis is performed.

7.7.2. State Assignment via Hidden Markov Model

The contour length change as a result of the transition from one protein state to another can be observed via a force change. Ideally, the state of the protein directly infers from the elongation of the spring. Single-molecule data from experiments at room temperature is inherently deteriorated by instrumental and thermal noise. The ultimate goal is to decipher the underlying states causing the observed data.

This requires to optimize model parameters that describe the data as accurately as possible. The model parameters are the emission probabilities

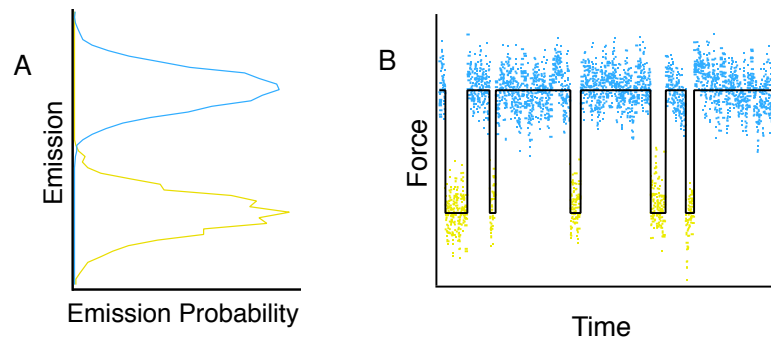


Figure 32 An example SMFS time trajectory of a sample protein fluctuating between two states. The two states are assumed to have each a different average extension. These distributions are called the emission probabilities (A). Example of a time trajectory (the colors correspond to different states.) and the assigned underlying states (black line).

(figure 32A) and the probabilities for the transition from one state into another. Simply speaking, the model compares the force values with the given emission probabilities and uses the transition probabilities to infer the hidden state of the protein at any time point. The parameter optimization is following an HMM. Ref. [SR12] gives details on the analysis of data obtained during passive mode experiments (section 6.1) using HMM. Furthermore, in complex situations, many states might project onto the same observable. Say, different folded states have an identical extension, but differ much in stability and thus in lifetime. HMM analysis is capable of distinguishing states that differ only in lifetime. Thus, single pair correlation analysis can be used to validate the results of HMM analysis in such complex scenarios, as well as, results involving short-lived states or noisy data [HW11].

7.7.3. Extracting Protein Un-/Folding Rates from *Jump Experiment Data*

Transition kinetics between states that are directly observable during *jump experiments* are eligible for direct lifetime analysis. One example is N_c unfolding at high force. In this case, the force change as a result of a transition is resolved. The *jump experiment* specific analysis procedures were developed and substantiated via kinetic simulations (as explained in section 7.2). The FLNa20 transition rates as reported in ref. [RMOR14] were used as simulation input in scenarios of SlyD absence. The kinematic simulation accounts for the elastic properties of the DNA-handles, but the kinetic model does not include thermal noise and hydrodynamic drag of the beads (figure 33).

Post-processing, as explained in section 7.7.1, was performed on the simulated data to validate the analysis methods that are presented in section 7.7.3 and following. The dumbbell step response correction (section 7.1) was tested accordingly. To do so, the force trajectory was cut into short

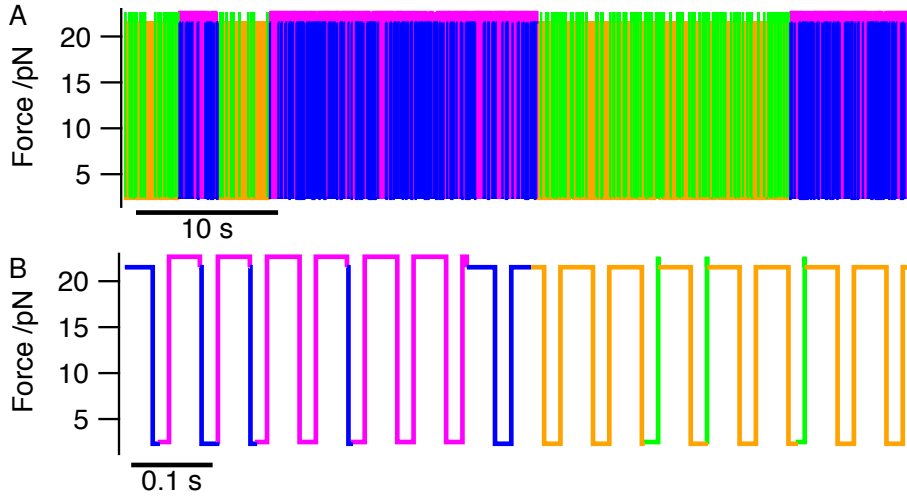


Figure 33 Kinetic simulations of *jump experiments* with the kinetic network of FLNa20. The un-/folding force trajectory shows multiple proline isomerization events (A). A zoom into the force trajectory (B). The switch of the un-/folding kinetic in the middle is due to proline isomerization. In kinetic simulations in contrast to experiments, the trajectory of states is known. Colors are assigned according to the state.

parts corresponding to the jump dwell times at different force levels. The start of each trunk of the trajectories was replaced with the consecutive data points to suppress the spikes that arise from the force jumps (section 7.1). The parts of the individual force level data were concatenated to form continuous time trajectories that are suitable for further analysis.

Figure 34 shows an example of a high force time trajectory after post-processing from a kinetic simulation. The lifetime of N_t here at 22 pN biasing force, are expected to be in the order of 10^{-9} s, well below the 10^{-3} s step response time of the dumbbell. In time trajectories like this, the N_t state is never resolved. The native cis-state and the unfolded states are assigned via HMM analysis. Lifetimes of the unfolded states cluster into two branches containing the long-lived U_t and the short-lived U_c events. A lifetime threshold is applied before the clusters are analyzed individually.

The lifetimes t_n are used to calculate the MLE^3 of the unfolding rate. The MLE is given by:

$$E_{MLE}(k_{N,U}(f_h)) = \frac{N}{\sum_{n=0}^N t_n} \quad (7.34)$$

The lifetimes of the native state at high force is directly observable. The

³ Maximum-likelihood estimation is a method of estimating the parameters of a statistical model. The parameter values are chosen such, that the likelihood of making a given observation is maximized. It was widely popularized by Ronald Fisher between 1912 and 1922 (used earlier by Carl Friedrich Gauss, Pierre-Simon Laplace, Thorvald N. Thiele, and Francis Ysidro Edgeworth) and proven in 1938 by S.S. Willks [Wil38]

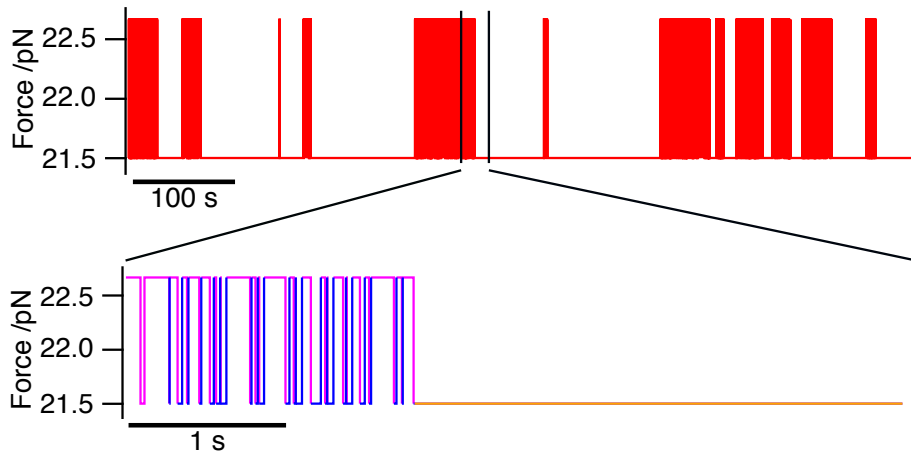


Figure 34 Simulated un-/folding trajectory after post-processing. The high force level of a two-state force *jump experiment* is shown. The lifetime of N_t at this force is too short to be detected. Thus, phases of FLNa20 in its trans conformation show long unfolded stretches. The zoom into the trace unveils several N_c unfoldings and visualizes the lifetime difference of U_c (blue) and U_t (orange). Colors in the zoom are assigned corresponding to the state.

lifetimes of the state, accounting for the lifetime in all force levels $\sum_j \tau_j$ is:

$$t_n = t_n \frac{\sum_j \tau_j}{\tau_h}$$

The nature of the orange state is different. From the *jump experiment*, it is not possible to distinguish whether the long-lived unfolded state is folding incompetent or its native state is too weak to be resolved. Fast refolding states (shown as blue dots in figure 48B) divide into two lifetime populations, shorter than a single jump cycle or longer. The refolding rate at the low force level F_l is calculated by the number of events in these lifetime clusters using equation 7.25.

The statistical error of the *MLE* as well as of the integrated lifetimes (figure 48B) are estimated by bootstrapping a confidence interval [Efr79] and [Efr87]. First, the data is resampled with replacement. From this sample, the *MLE* of the off rate and the cumulative lifetimes are calculated. The integrated lifetime probabilities for the lifetimes of the original sample are interpolated. This routine is repeated, (throughout this work 1000 times unless mentioned otherwise) to gather statistics. The individual errors are estimated using the 16 % and 68 % quantile of the resampled values.

7.8. Numerical Study to Determine Suitable Jump Experiment Parameter

The *jump experiment* protocol allows versatile manipulation of protein conformation dynamics. Therefore it is crucial to choose the experimental pa-

rameters suitable to observe specific transitions. The kinetic simulations that are shown in the following subsections were used to design the FLNa20 experiments that are presented in part III. Kinetic simulations (as explained in section 7.2) using the FLNa20 kinetic network (with transition rates as in Ref. [RMOR14]) as input were conducted to choose suitable *jump experiment* parameter.

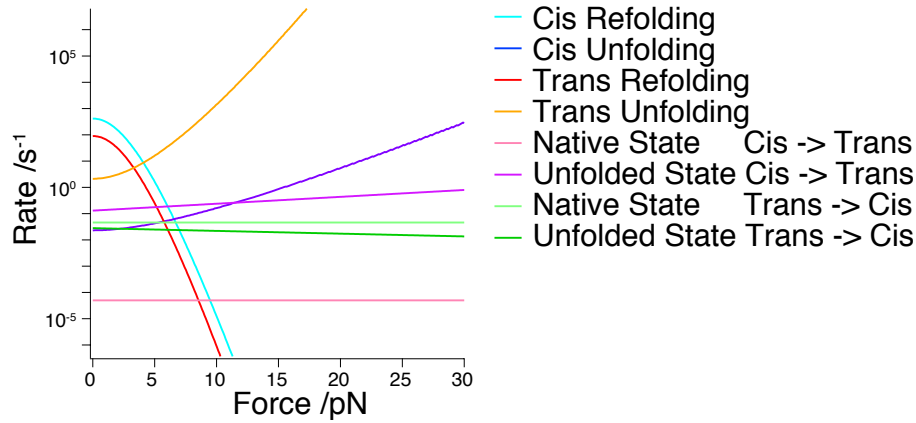


Figure 35 Force-dependent model of FLNa20 transition rates. The kinetic parameters used for the force-dependent transition rates are taken from [RMOR14]

Figure 35 shows the force-dependent FLNa20 transition rates in the absence of SlyD that were used as simulation input [RMOR14].

7.8.1. Un-/Folding Kinetics Measured using Fast Force Jump Experiments

First, in a set of *jump experiment simulations*, the un-/folding kinetics of FLNa20 under force-jump conditions was studied. The results of that un-/folding study on FLNa20 substantiate the fast force-jump protocol and was used to explore possible interactions of FLNa20 and SlyD as presented later in section 9.2 and section 9.4. The methods to observe un-/folding rates via *jump experiments* (described in section 7.5) were used on the simulated data. Reported force-dependent transition rates [RMOR14] and experimental parameters that are favorable for *jump experiments* were used as input for the simulations (explained in section 7.2). The jump dwell times were kept at $\tau_l = 20$ ms and $\tau_h = 40$ ms. The AOD positions of the jump levels were randomly chosen from a normal distribution within $x(AOD_l) = 300 - 400$ nm and $x(AOD_h) = 500 - 800$ nm. Folding was observed at $0.2 - 5$ pN and unfolding at $12 - 28$ pN. After post-processing that is needed for experimental data (section 7.7.1), the unfolding rate of N_c was determined by lifetime analysis as described in section 7.7.3.

Figure 36 shows the force-dependent unfolding rates and the corresponding residuals. For a set of 100 independent simulations, the extracted rate deviated $(-0.01 \pm 0.04) \text{ s}^{-1}$ from the input. The residual distribution was

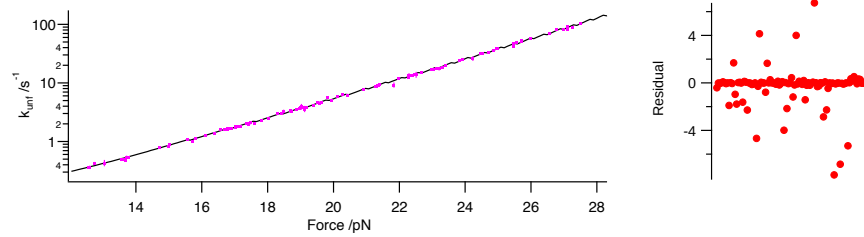


Figure 36 Force-dependent unfolding rates of N_c determined by dwell time analysis. The residuals are normally distributed. The dwell times are $\tau_l = 20$ ms and $\tau_h = 40$ ms.

compared to a normal distribution via the Kolmogorov-Smirnov-Test⁴. Since the critical value was bigger than P , the folding rates were considered normally distributed (see table 1).

Table 1 Results of the Kolmogorov–Smirnov-test for normally distributed residuals of unfolding rates extracted from kinetic simulations. The Kolmogorov–Smirnov statistic D is smaller than the critical value, thus the residuals of the extracted rates are considered normally distributed. With the variance exceeding the deviations from the input, the analysis appears bias-free.

α	0.05
D	0.0823585
Critical	0.134028
P	0.244

The force-dependent folding rate was determined using equation 7.25 on a set of simulated *jump experiments* after post-processing that is needed for experimental data (section 7.7.1).

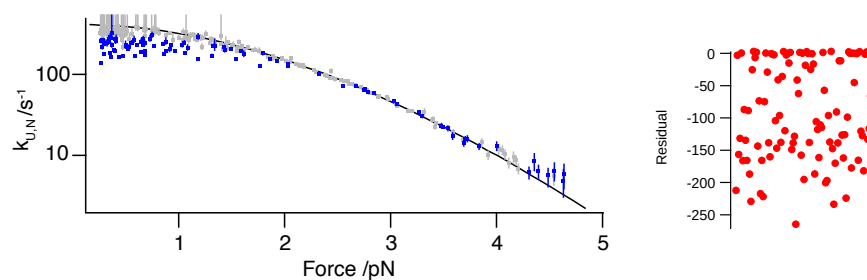


Figure 37 Force-dependent folding rates of U_c determined as explained in section 7.5. The black line shows the folding rate used as input for the simulations. The blue points indicate folding rates after post-processing (section 7.7.1). 20 of the 100 values are within expectation. The residuals show a negative bias (right panel). As a reference, kinetic simulations with ideal step response are shown in gray.

⁴ The Kolmogorov–Smirnov test is a non-parametric test of the equality of continuous, one-dimensional probability distributions that can be used to compare a sample with a reference probability distribution. It is named after Andrey Kolmogorov and Nikolai Smirnov.

Figure 37 shows the results from direct analysis of simulated data as well as results after hydrodynamic step-response correction (section 7.1). Measured rates and the input folding rate are shown in figure 37 (left). After application of the hydrodynamic correction, the values showed a negative bias of $(-90 \pm 77) \text{ s}^{-1}$. Only 20 % of the analyzed simulations showed a folding rate within the corresponding one σ error. Kinetic simulations without the hydrodynamic step response correction (section 7.1) did not show such a bias (figure 37). The rates measured in the *simulations* as shown in figure 37 are prone to missed unfolding events. An unresolved folding and unfolding cycle is misinterpreted as a long unfolded lifetime. The missed events could be quantified by using (7.2) with $t = t_{\text{cut off}}$. The unresolved folded lifetime probability is given by $p_{\text{missed w/o effect}} = 1 - \frac{n_{\text{long}}}{n_{\text{tot}}}$, thus the underestimation of refolding within the first low force dwell can be calculated. The correction factor for long unfolded lifetimes is given by:

$$C = \frac{1 - \exp(-k\tau_h)}{(1 - \exp(-kt_{\text{cut off}}))(1 - \frac{n_{\text{long}}}{n_{\text{tot}}})} \quad (7.35)$$

The probability of an unresolved folding event did not decrease the number of long unfolded lifetimes. Figure 38 shows the results of a *simulation* set after application of the hydrodynamic step response correction and accounting for missed events.

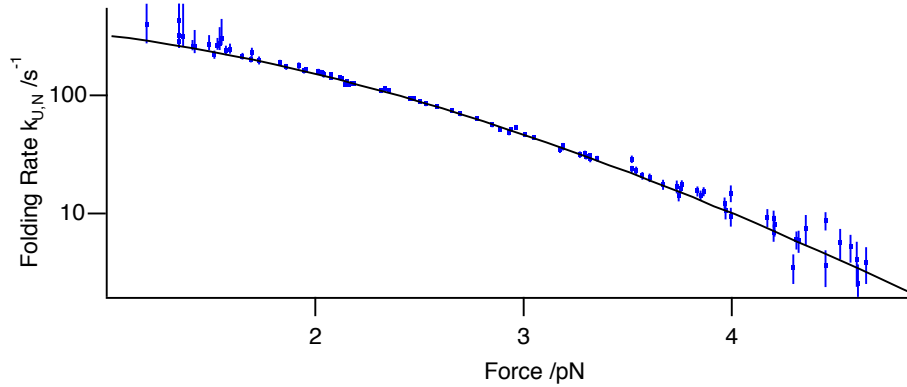


Figure 38 Force-dependent folding rates of U_c determined as explained in section 7.5. The black line shows the folding rate that was used as input for the simulations. The blue points indicate folding rates after post-processing as described in section 7.7.1 with an additional correction for missed unfolding events. 64 of the 100 values are within expectation.

After correction for hydrodynamic drag and missed events, the folding rate that was used as input for the *simulation* was within expectation for 64 of the 100 *simulations* (section 7.7.3). The data obtained in the *simulations* used to produce the results are shown in figure 38, a $\tau_1 = 20 \text{ ms}$ was used as input. At lower folding forces F_l than shown, all folding events occur within the first low force dwell time. Thus, equation 7.25 used for analysis yields infinite folding rates.

7.8.2. Kinetics of Unfolded Proline Cis-Trans Isomerization Measured using Fast Force Jump Experiments

The present section presents the *simulations* that were used to determine suitable *jump experiment* parameters to determine FLNa20 folding. Unlike for FLNa20 un-/folding, there is no direct SMFS signal corresponding to proline isomerization. Although the cis- and trans-state of p2225 in FLNa20 during a *jump experiment* can be assigned due to different un-/folding kinetics (as explained in section 7.6), it remains hidden whether the isomerization occurred in the native or the unfolded state. The kinetic simulations of *jump experiments* on FLNa20 demonstrate that it is possible to determine the native as well as unfolded *cis-trans*-isomerization rates individually.

Cis-FLNa20 population probabilities of the native and unfolded states were determined via the un-/folding rates. The native state *cis-trans*-isomerization $k_{N_c, N_t} \approx 10^{-5} \text{ s}^{-1}$ compared to the unfolded $k_{U_c, U_t} \approx 10^{-1} \text{ s}^{-1}$ was neglected to calculate the unfolded force-dependent *cis-trans*-isomerization rate.

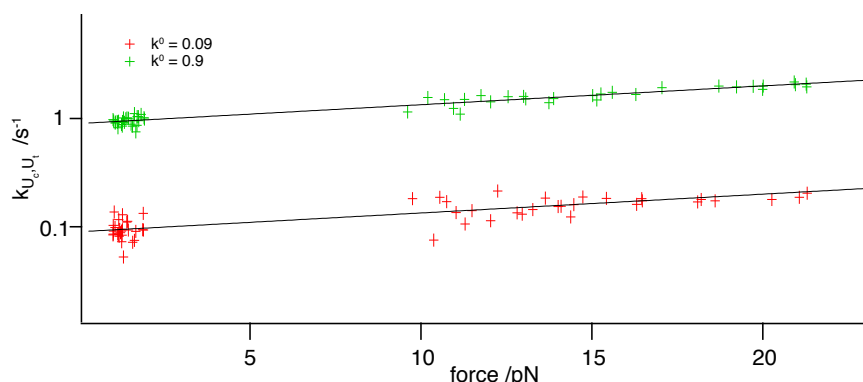


Figure 39 Force-dependent *cis-trans* isomerization rate $k_{U_c, U_t}(F)$ extracted from simulated *jump experiments*. Simulation of the non-enzymatic FLNa20 kinetic network (red) and tenfold increased unfolded *cis-trans* isomerization kinetics k_{U_c, U_t}^0 (green). Black lines: simulation input. The simulation length is kept constant, thus faster kinetics result in better statistics and less scattering of the values.

Figure 39 shows unfolded *trans-cis*-isomerization rates that were determined via equation 7.26 and equation 7.27 using simulated *jump experiments*. The obtained unfolding rates are consistent with the simulation input.

7.8.3. Kinetics of Unfolded Proline Trans-Cis Isomerization Measured using Fast Force Jump Experiments

The present subsection shows the *simulations* that were used to find a protocol to measure unfolded FLNa20 *trans-cis*-isomerization. Two force level *jump experiments* are typically conducted such that the N_t state is not re-

solved. For FLNa20, the long unfolded lifetimes are assigned to the p2225 trans-state. During a *jump experiment*, FLNa20 eventually refolds at low force, but the contour length change is not resolved. The N_t lifetime at high force is too short to be detected. Thus, the protein state population probabilities of trans-FLNa20 cannot be directly observed. Furthermore, the native state *trans-cis*-isomerization contributes considerably to $k_{t,c}^{app}$. Therefore, the *trans-cis*-isomerization rate of unfolded FLNa20 k_{U_t,U_c} cannot be determined via a single *jump experiment*.

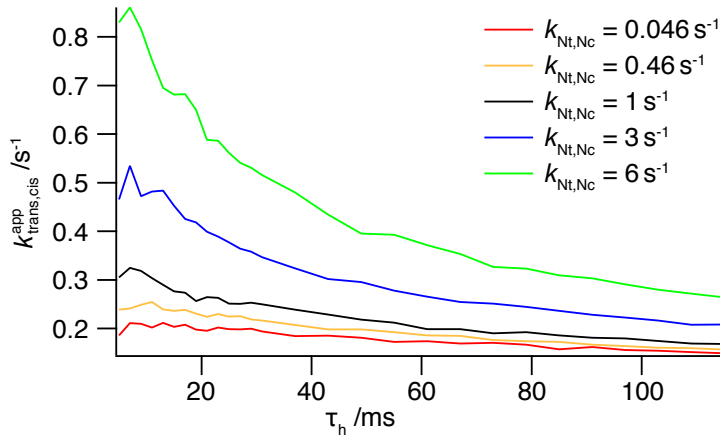


Figure 40 Apparent *trans-cis*-isomerization rate $k_{t,c}^{app}$ over high force jump dwell time τ_h . Different native state *trans-cis*-isomerization rates k_{N_t,N_c} are used as simulation input. $k_{t,c}^{app}$ is bigger for higher k_{N_t,N_c} values, and the $\frac{1}{\tau_h}$ component is more pronounced.

Figure 40 shows the τ_h -dependent, apparent *trans-cis*-isomerization rate $k_{t,c}^{app}$ for non-enzymatic (shown in red) and catalyzed native state *trans-cis*-isomerization. Considering *trans-cis*-isomerization only at the low force level F_l , $k_{t,c}^{app}(F_l)$ is τ_h independent. Therefore only $k_{U_t,U_c}(F_h)$ contributes to $k_{t,c}^{app}(\tau_h)$. *Jump experiments* with identical parameters other than τ_h can be used to determine $k_{U_t,U_c}(F_h)$.

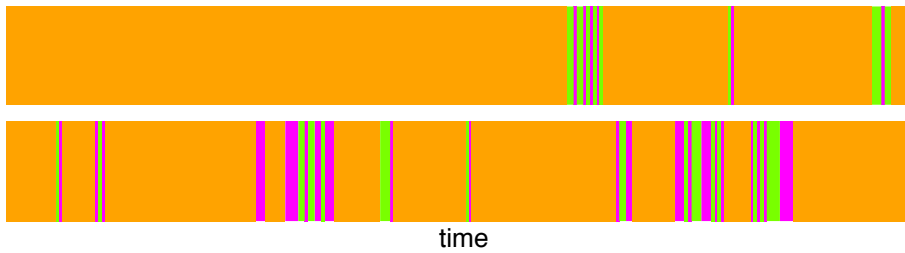


Figure 41 High force protein state trajectories after post-processing (section 7.7.1) as a simulation outcome. The *jump experiment* parameters but the jump dwell time at high force τ_h are kept identical. In the upper trajectory, τ_h is five times as long as in the lower one. Lifetimes of the protein states depend on τ_h . Colors are assigned to the protein states U_t (orange), N_c (pink) and U_c (green).

Figure 41 shows FLNa20 state trajectories obtained during simulated *jump experiments* that differ only in τ_h by a factor of five. The proline isomerization behavior appears τ_h -dependent. Independent of τ_h , every jump dwell

time at low force starts in the U_t state. As a consequence, if the apparent proline isomerization rate is divided into an apparent proline isomerization rate under high force $k_{t,c}^{\text{app},h}$ and under low force $k_{t,c}^{\text{app},l}$, the later one is τ_h independent (see equation 7.31). Using equation 7.32, the unfolded state FLNa20 *trans-cis*-isomerization rate k_{U_t,U_c} can be calculated as:

$$k_{U_t,U_c}(F) = \frac{T_{\text{long}} \cdot k_{U_t,U_c}^{\text{app},\text{long}} - T_{\text{short}} \cdot k_{U_t,U_c}^{\text{app},\text{short}}}{(\tau_h^{\text{long}} - \tau_h^{\text{short}})} \quad (7.36)$$

with the jumping period T , the jump dwell time at high force τ_h and the apparent *trans-cis*-isomerization rate $k_{t,c}^{\text{app}}$. Errors are calculated by interval propagation to:

$$\sigma_{k_{U_t,U_c}} = \frac{T_{\text{long}}}{(\tau_h^{\text{long}} - \tau_h^{\text{short}})} \sigma_{k_{U_t,U_c}^{\text{app},\text{long}}} + \frac{T_{\text{short}}}{(\tau_h^{\text{long}} - \tau_h^{\text{short}})} \sigma_{k_{U_t,U_c}^{\text{app},\text{short}}} \quad (7.37)$$

Common values for τ_h in *jump experiments* likely are 5 ms, 10 ms, 20 ms or 50 ms in combination with a τ_l that is suitable to measure U_c folding, 10 ms or 20 ms.

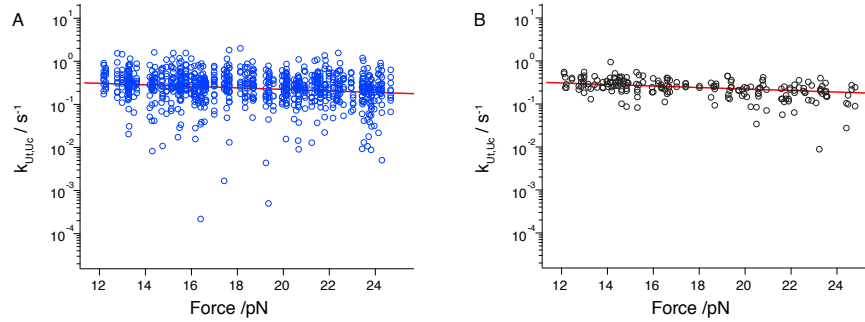


Figure 42 Force-dependent unfolded *cis-trans*-isomerization rates determined from simulated *jump experiments* using equation 7.36. The *cis-trans*-isomerization rate that was used as simulation input is shown as red lines. Simulations with $F_l = 1$ pN and $\tau_l = 10$ ms and $\tau_h \in \{5, 10, 20, 50\}$ were conducted. A shows $k_{U_t,U_c}(F_h)$ determined from all simulations. For B only simulations with $\tau_h \in \{5, 10\}$ ms in combination with $\tau_h = 50$ ms were analyzed.

Figure 42 shows force-dependent *trans-cis*-isomerization rates calculated from simulated *jump experiments* using equation 7.36. Simulations using $F_h \in (12, 24)$ pN, $\tau_h \in \{5, 10, 20, 50\}$ ms, $F_l = 1$ pN and $\tau_l = 10$ ms were conducted. Figure 42A shows k_{U_t,U_c} determined from all simulations. Whereas figure 42B only shows k_{U_t,U_c} calculated from simulations with $\tau_h^{\text{short}} \leq 10$ ms and $\tau_h^{\text{long}} = 50$ ms. First, the error as determined via equation 7.37 is proportional to $(\tau_h^{\text{long}} - \tau_h^{\text{short}})^{-1}$. And second, the τ_h -dependent $k_{t,c}^{\text{app}}$ considerably changes between 10 ms and 50 ms. The variation of observed *trans-cis*-isomerization rates relates considerably to the *jump experiment* parameter.

Figure 43 shows the force-dependent FLNa20 proline isomerization rates $k_{U_t,U_c}(F)$ that were determined from data obtained during *jump experi-*

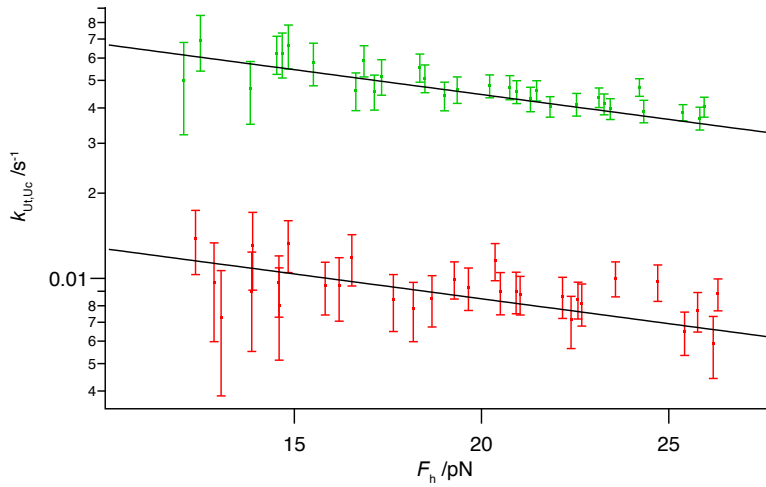


Figure 43 Force-dependent unfolded FLNa20 *trans-cis*-isomerization rates obtained during simulated *jump experiment*. *Jump experiment* parameter $F_j = 0.6$ pN, $\tau_j = 10$ ms, short jump dwell time at high force $\tau_h^s = 10$ ms and long $\tau_h^l = 50$ ms are used for all simulations. Two different *trans-cis*-isomerization rates (black lines) k_{U_t,U_c}^0 0.019 s $^{-1}$ (as red dots) and 0.9 s $^{-1}$ (as green dots) are used as simulation input. Simulated time is in the order of 10^5 s for each experiment.

ments using suitable experimental parameters, such that the $k_{t,c}^{\text{app}}$ rates for *jump experiments* with different τ_h differ considerably (as in figure 41). The non-enzymatic unfolded FLNa20 *trans-cis*-isomerization rate 0.019 s $^{-1}$ was used as simulation input (shown as red dots), as well as an enzymatic 0.09 s $^{-1}$ (shown as black dots). The difference in *cis-trans*-isomerization rates is well resolved in typical *jump experiments*.

7.8.4. Kinetics of Native State Proline Trans-Cis Isomerization Measured using Fast Force Jump Experiments

The native state of trans-FLNa20 is not observed during a typical *jump experiment*. Nevertheless, with the unfolded *trans-cis*-isomerization, the native state FLNa20 *trans-cis*-isomerization can be observed via *jump experiments*. In the present subsection, *jump experiment simulations* are used to demonstrate how.

The N_t state is not resolved during two force level *jump experiments*. Assuming the force dependence of native FLNa20 *trans-cis*-isomerization, e.g. like the Bell model, it is possible to calculate k_{N_t,N_c} for an individual *jump experiment*. A series of *jump experiments* with different τ_h was simulated to measure $k_{N_t,N_c}(F)$. The sensitivity for native state *trans-cis*-isomerization was tested by using different k_{N_t,N_c} as simulation input. Figure 40 shows the τ_h -dependent apparent *trans-cis*-isomerization for different k_{N_t,N_c} , as obtained via simulations. Assuming, that the unfolded *trans-cis*-isomerization rate under high force is identical to the apparent *trans-cis*-isomerization rate under low force $k_{U_t,U_c}(F_h) = k_{t,c}^{\text{app},l}$ (equation 7.31), the apparent *trans-*

cis-isomerization rate $k_{t,c}^{\text{app}}$ is τ_h independent. If high k_{N_t,N_c} is used as input for *jump experiment* simulations, the apparent *trans-cis*-isomerization rate under low force $k_{t,c}^{\text{app},l}$ is bigger than $k_{U_t,U_c}(F_h)$. The $k_{t,c}^{\text{app}}$ observed for short τ_h is bigger than for long τ_h , e.g. shown as a green line in figure 40. After extracting the force-dependent unfolded FLNa20 *trans-cis*-isomerization rate $k_{U_t,U_c}(F)$, equation 7.26 was used to calculate the native state *trans-cis*-isomerization rate.

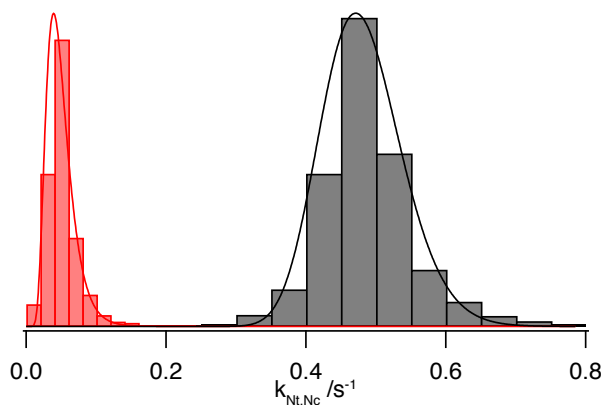


Figure 44 k_{N_t,N_c} histograms obtained during *jump experiment* simulations. After determination of $k_{U_t,U_c}(F)$ equation 7.26 is used to measure k_{N_t,N_c} . Lognormal distribution functions are shown as solid lines. Simulation input: $k_{N_t,N_c} = 0.1 \text{ s}^{-1}$ in red and 0.5 s^{-1} in black.

Figure 44 shows native state *trans-cis*-isomerization rate histograms obtained during simulations with different k_{N_t,N_c} .

7.8.5. Kinetics of Native State Proline Cis-Trans Isomerization Measured using Fast Force Jump Experiments

FLNa20 *cis-trans*-isomerization in the unfolded state can be observed during *jump experiments* without considering native state proline isomerization. Native state *cis-trans*-isomerization (10^{-5} s^{-1}) is intrinsically multiple orders of magnitude slower than unfolded state isomerization (10^{-2} s^{-1}) and thus does not considerably contribute to $k_{c,t}^{\text{app}}$ under equilibrium conditions.

During *jump experiments*, protein states population probabilities can be shifted with respect to in-equilibrium conditions. Figure 45 shows F_h -dependent non-enzymatic unfolded FLNa20 *cis-trans*-isomerization rate k_{U_c,U_t} obtained via *jump experiment* simulations using jump dwell times $\tau_h = 20 \text{ ms}$ and $\tau_l = 10 \text{ ms}$. The measured *cis-trans*-isomerization rate k_{U_c,U_t} show systematic deviation from the simulation input for F_h smaller than 10 pN. Since k_{U_c,U_t} is calculated under the assumption: $p_{N_c} \cdot k_{N_c,N_t} \ll p_{U_c}(F) \cdot k_{U_c,U_t}(F)$, the deviations origin from k_{N_c,N_t} contribution. Experimental *jump experiment* parameters can be chosen such that *jump experiments* are sensitive to

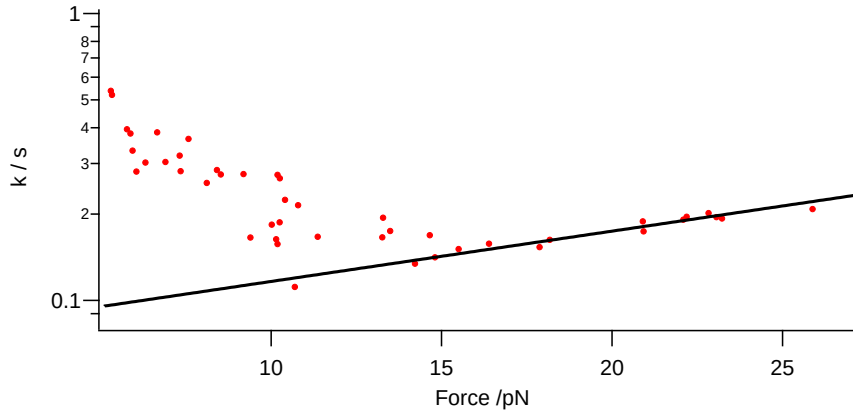


Figure 45 Non-enzymatic force-dependent, unfolded FLNa20 *cis-trans*-isomerization rates $k_{c,t}^{\text{app}}$ obtained via *jump experiment* simulations. The jump dwell times are chosen such, that N_c population is increased: $\tau_h = 10$ ms and $\tau_l = 20$ ms. For F_h smaller than 10 pN, the measured k_{U_c, U_t} values are higher than the simulation input, due to the non-negligible contribution of k_{N_c, N_t} .

k_{N_c, N_t} . During such *jump experiments*, FLNa20 is dominantly found in its native state, thus $k_{c,t}^{\text{app}}$ is slow. The simulated time of each *jump experiment* was 1.5×10^6 s to obtain a sufficient number of isomerization events.

Jump experiments can be used to drastically change population probabilities and thus promote or suppress transition paths. In particular, if the native folded *cis*-state N_c population probability is considerably enhanced ($\gg 0.99$), the native state *cis-trans*-isomerization contributes considerably to $k_{c,t}^{\text{app}}$.

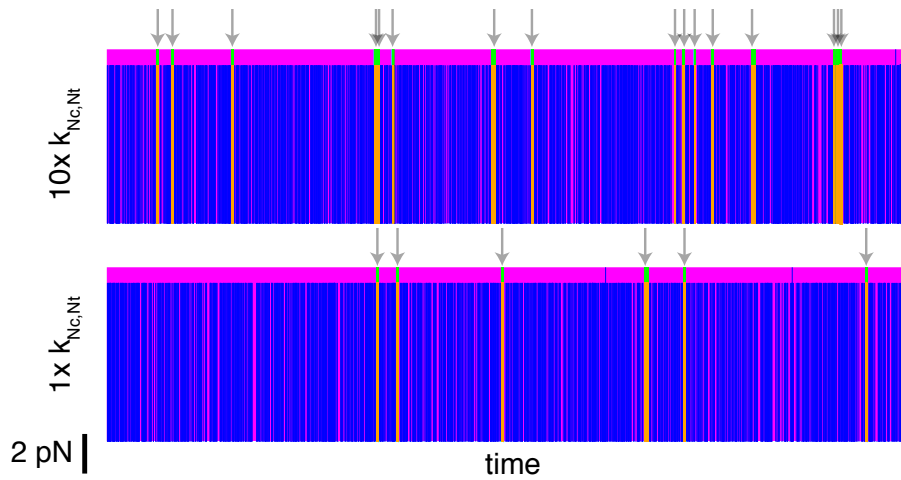


Figure 46 Un-/folding force trajectories obtained from simulated *jump experiments* with different native state *cis-trans*-isomerization rates k_{N_c, N_t} . Input k_{N_c, N_t} for the top simulation is ten times bigger with respect to the bottom. The other input parameters remain identical. The proline isomerization obtained during *jump experiments* shows native state *cis-trans*-isomerization sensitivity. The different colors correspond to the FLNa20 states N_c (pink), U_c (blue), N_t (green) and U_t (orange). The gray arrows indicate trans-states.

Figure 46 shows simulated FLNa20 *jump experiments* to $F_h \approx 10$ pN. During the simulations, multiple cis (in pink and blue) and trans phases (in green and orange, highlighted with gray arrows) were observed. The input parameters used to create the bottom trace correspond to FLNa20 in absence of SlyD. The native state *cis-trans*-isomerization rate used as simulation input to create the top trace was tenfold increased. While the cis and trans-FLNa20 un-/folding kinetics appear similar, FLNa20 cis lifetimes are shorter in the top case.

Apparent *cis-trans*-isomerization rates $k_{c,t}^{\text{app}}$ obtained via simulated *jump experiment* time trajectories depend linear on k_{N_c, N_t} . For a catalyzed unfolded state *cis-trans*-isomerization rate k_{U_c, U_t} of 1.5 s^{-1} and F_h of 4.7 pN the proportionality factor $p(N_c)$ is close to one.

7.8.6. Summary

In numerical studies, the results for FLNa20 unfolding rates appeared robust. Within wide parameter variations, the calculated errors were in accord with the corresponding simulation input. The mean of the determined unfolding rate residuals was zero within error (figure 36). In a Kolmogorov–Smirnov-Test the residuals were confirmed to be normally distributed (table 1). The analysis methods that are provided in section 7.7.3 give a bias-free estimator in the parameter space investigated.

Extracting folding rates from simulations based on a maximum-likelihood approach (as derived in section 7.5) showed a pronounced systematic underestimation of the folding rate (figure 37). Despite that general finding, for FLNa20 there were sweet spots in the *jump experiments* parameter space as well in which the folding rate was measured precisely ($F = 2-3$ pN in figure 37).

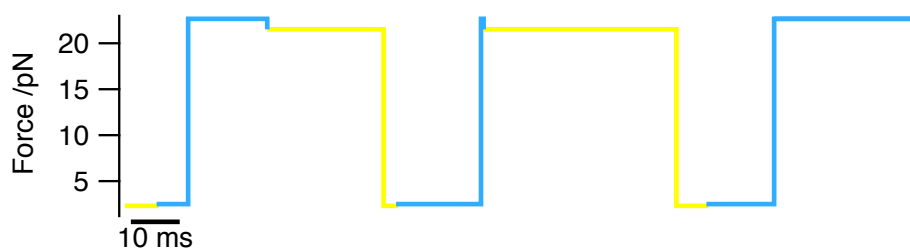


Figure 47 Artifact from a missed un-/folding event. Two consecutive unfoldings with the second being shorter than the dead time of the *jump experiment* (0.5 ms). The two events of folding within one jump to low force would appear as one long unfolded lifetime.

Figure 47 shows how a missed un-/folding cycle is misinterpreted as a long unfolded lifetime. The step response of the dumbbell during a *jump experiment* is finite, due to the hydrodynamic drag of the beads (figure 19). To avoid artifacts, force data measured after settling of the beads was extrapolated to the time of the force jump. This leads to a blind period of 0.5 ms

after the force jump. Unfolding of N_c within that dead time skews the lifetime distribution and thus, the folding probability is biased. Figure 37 shows such artifacts originating from missed events under low forces, in folding rates determined using simulated data. The number of missed events was calculated (as explained in section 7.4.1) to correct the fraction of unfolded lifetimes longer than one jump period. Using this correction, the calculated folding rates and errors were consistent with the simulation input (figure 38).

Simulations showed as well, that unfolded isomerization rates k_{U_c, U_t} and k_{U_t, U_c} , that are prone to enzymatic proline isomerization in this model, can be obtained consistently and independent of the native state proline isomerization rate during *jump experiments*. The apparent proline isomerization rates $k_{c,t}^{app}$ and $k_{t,c}^{app}$ showed distinctive dependence from *jump experiment* parameters for relative isomerization rates $\frac{k_{N_c, N_t}}{k_{U_c, U_t}}$ and $\frac{k_{N_t, N_c}}{k_{U_t, U_c}}$. Variation in measured rates considerably depends on the *jump experiment* parameters.

From a set of *jump experiments*, the native state *trans-cis*-isomerization rate k_{N_t, N_c} can be obtained and with previous knowledge of the unfolded state *trans-cis*-isomerization rate. Notably, using $F_h < 10$ pN as simulation input for *jump experiments*, the dominating proline isomerization pathway was changed from unfolded to native state isomerization. With the unfolded isomerization rate k_{U_c, U_t} , it is possible to determine consistent k_{N_c, N_t} values.

Part III

Results

8. The FLNa20 Kinetic Network in the Presence of SlyD

The present chapter provides an overview of a typical *jump experiment* on the filamin protein domain FLNa20 with and without the enzyme SlyD. During N–C–terminal *jump experiments*, the FLNa20 (chapter 3) transition rates were measured. The *jump experiment* protocol as described in chapter 7 was used to also obtain enzymatic proline isomerization of FLNa20 p2225 in the presence of SlyD. The results of specific *jump experiments* to determine un-/folding and also proline isomerization rates are presented in the subsequent chapters.

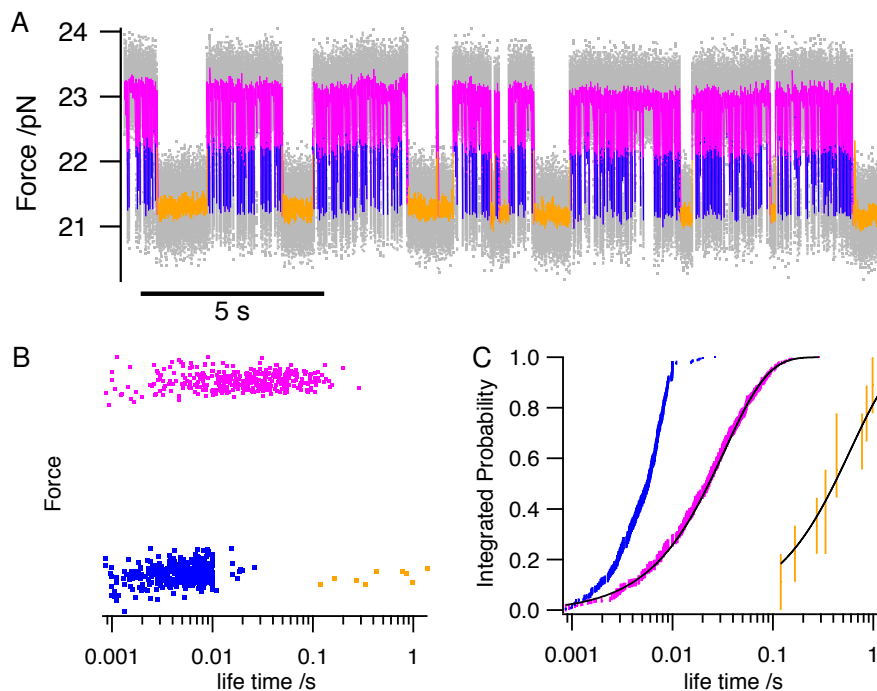


Figure 48 Data collected during a two force level *jump experiment* in the presence of 1 μM SlyD. The un-/folding force trajectory shown in (A) corresponds to the high force level and was created as explained in section 7.7.1. *Jump experiment* parameters: $\tau_h = 10$ ms, $\tau_l = 60$ ms, $F_h = 23$ pN and $F_l = 1.1$ pN. Native (in pink) and unfolded (cis in blue and trans in orange) states are assigned via HMM analysis. The unfolded state shows distinct kinetics of two different states with identical mean force. They are separated using a threshold chosen within the lifetime gap in the force over lifetime scatter plot (B). (C) shows integrated lifetime histograms.

Figure 48 shows an example un-/folding force trajectory that was created from the high force level obtained during a *jump experiment*, (using the post-processing procedure described in section 7.7.1). The native and unfolded states have been identified via HMM analysis. In a force vs lifetime plot (figure 48C), the unfolded state population divides into two clusters.

The points were classified into cis and trans using a threshold. The short-lived unfolded state (colored in blue) subdivides into events that either refold within the first or in the second dwell time at low force. The lifetimes of the short-lived unfolded state are dominated by the initial dead times τ_{Dead} (see section 7.4.1), thus showing a non-exponential distribution. The lifetimes of the long-lived unfolded state (right cluster in orange) are much longer than τ_h . Thus the lifetimes are exponentially distributed, due to stochastic folding (figure 48B). Lifetime analysis for the natively folded state (in pink) and long-lived unfolded state (in orange) was performed to determine the rate out of state $i \in [N, U_{\text{long}}]$ at high force $k_{i,x}(F_h)$.

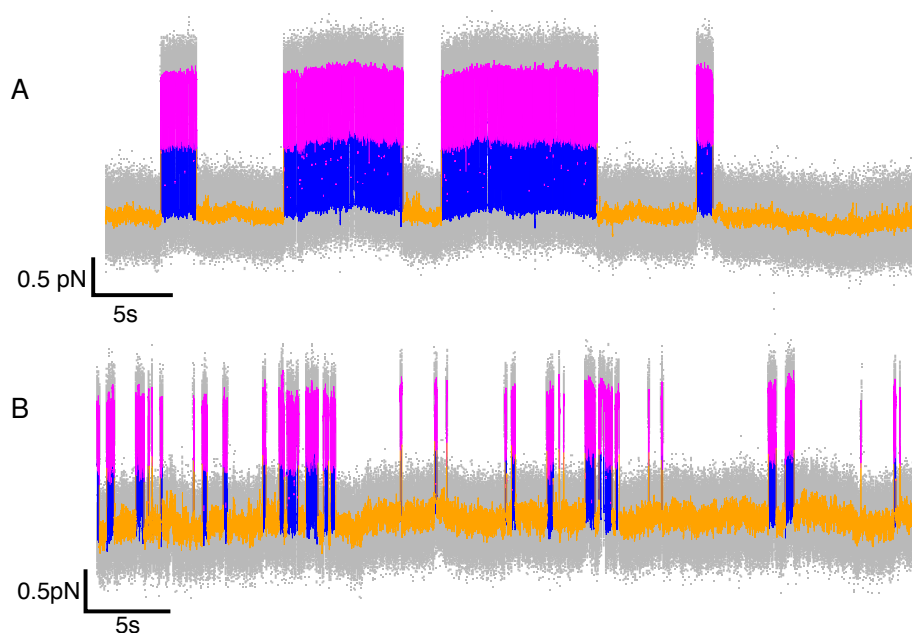


Figure 49 High force level un-/folding force trajectory obtained during *jump experiments* after post-processing (section 7.7.1). The time traces are sections of the same length. The experiments share comparable *jump experiment* parameters: $\tau_h = 10$ ms, $\tau_l = 10$ ms, $F_h = 26.2$ pN and $F_l = 1.2$ pN. In the absence of SlyD: (A) and in the presence of $1 \mu\text{M}$: (B). The color of smoothed data for folded and unfolded states were assigned by HMM. Proline isomerization phases were separated using a time threshold. Gray: Full bandwidth data. Pink: Cis folded, Blue: Cis unfolded, Orange: Trans unfolded. The un-/folding force trajectory shows that SlyD affects the proline isomerization kinetics.

Un-/folding force trajectories at the high force level for different SlyD concentrations show different proline isomerization behavior (figure 49). The trans phases (orange) appear more often and of a shorter lifetime in the presence of SlyD. The proline isomerization rate in absence of SlyD is slow, and trans phases under *jump experiment* conditions are rare. In the subsequent chapters, studies of FLNa20 un-/folding kinetics (chapter 9) are presented, as well as investigations of SlyD-dependent p2225 proline isomerization (chapter 10).

9. Un-/Folding Kinetics of Filamin A Domain 20

In the following, a detailed investigation of FLNa20 un-/folding kinetics under various SlyD concentrations is presented. One of the main goals of this work is the characterization of hypothesized stable complexes formed by FLNa20 and SlyD. Stable binding of SlyD would induce changes in the FLNa20 un-/folding behavior. The bimodal un-/folding kinetics of the cis and the trans-state can be exploited to observe proline isomerization [RMOR14]. Detection of isomerization transitions in constant distance experiments is based on the difference in the lifetime of the N_c and N_t states. Enzymatic proline isomerization kinetic is fast compared to the frequency at which un-/folding occurs. In particular, back and forth isomerization occurs within a single unfolded lifetime during constant distance experiments. This results in a non-negligible fraction of the proline isomerization events being undetected by observing the un-/folding kinetics. Therefore the fast force-jump-protocol (developed in chapter 7) was used to speed up the un-/folding kinetics and manipulate population probabilities of conformational states as well as the transition *pathways*. During typical *jump experiments*, transitions between the folded and unfolded state of trans-FLNa20 are not observed. Experiments to measure the un-/folding rates of cis-FLNa20 in a force-jump protocol are presented in the following sections.

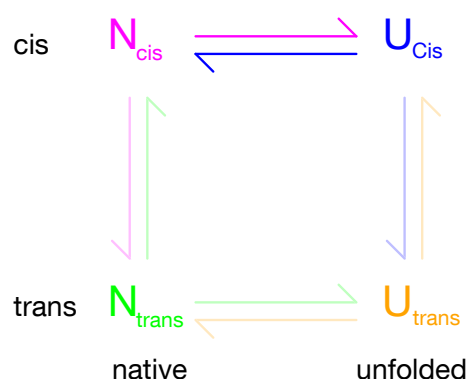


Figure 50 Kinetic network of FLNa20 states. The cis,un-/folding transitions that are observable during a typical *jump experiments* are shown in bright colors.

Figure 50 shows the kinetic network that is created by the potential FLNa20 states in the presence of SlyD. The SlyD bound states are potential kinetic traps that might slow down folding [Kie95].

9.1. Unfolding Kinetics of Cis-Filamin A 20

The SlyD-dependent complex kinetic multistate network of FLNa20 was studied in this work. In the first set of *jump experiments*, the SlyD dependent folding was studied. The established single-molecule force spectroscopy protocols appeared to be insufficient for detailed studies of complex kinetic networks that include cases like enzymatic isomerization, where the isomerization rate is similar to the un-/folding kinetics. *Jump experiments* were designed to overcome the described limitation. The *jump experiment* unfolding results were used to validate the jumping protocol with constant distance data. Experiments that investigate the unfolding behavior of N_c are presented in the following section.

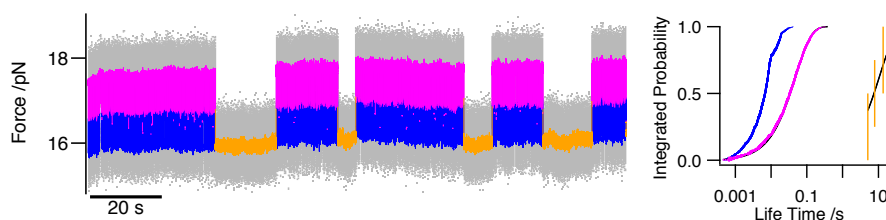


Figure 51 Example of an un-/folding force trajectory (left panel) created from the high force level of a two force level *jump experiment*. The color of smoothed data for folded and unfolded states was assigned using HMM. Proline isomerization phases are separated using a time threshold. The integrated lifetime histogram (right panel) with bootstrap errors and a single exponential cumulative lifetime. *Jump experiment* parameters: $\tau_h = 10$ ms, $\tau_l = 10$ ms, $F_h = 17.7$ pN and $F_l = 0.9$ pN. Gray: Full bandwidth data. Pink: Cis native, Blue: Cis unfolded, Orange: Trans unfolded.

A typical un-/folding high force time trajectory after post-processing is shown in figure 51. The FLNa20 states were assigned by applying HMM analysis on the data. The U_t and the U_c lifetimes are separated by almost two orders of magnitude. The transition rates from U_c to N_c and U_t to N_t were calculated from the lifetime distributions as explained in section 7.5. The cumulative lifetime histograms (colored dots in the right panel in figure 51) and exponential probability functions with the measured rates (black lines) are consistent. The probability function with the unfolding rate of N_c is mostly covered by the experimental lifetime histogram. The observed unfolding rate of N_c in this experiment is $(20.1 \pm 0.5) s^{-1}$ at 17.7 pN. The unfolding rate of N_c was observed during *jump experiments* covering a wide F_h range of 5–27 pN. The lower force level was kept at (0.6 ± 0.2) pN. The jump dwell times were kept in the range of 5–50 ms during the experiments.

Force-dependent unfolding rates measured via *jump experiments* were analyzed applying equation 7.34 after post-processing of the raw data as explained in section 7.7.1. The results were compared to rates measured in constant distance mode (figure 52). It is important to note, that the passive mode¹ and the *jump experiments* were conducted on different optical tweezers. The samples originated from different preparation batches. The

¹ The setup used for the passive mode experiments was designed by Christof Gebhardt (for details, see [Geb09])

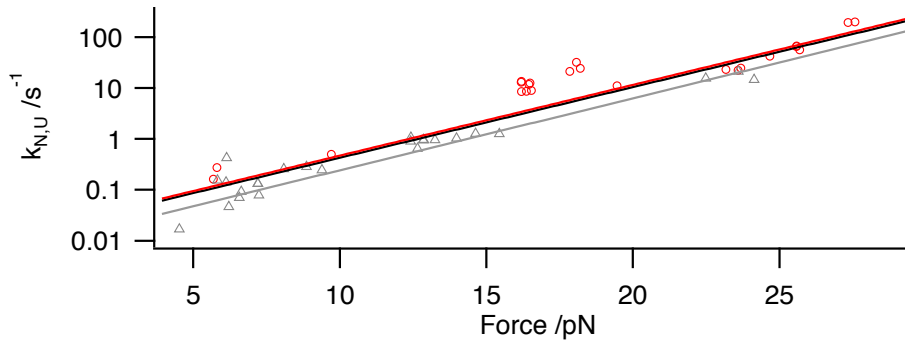


Figure 52 Force-dependent unfolding of FLNa20 obtained during *jump experiments* (red) and passive mode experiments. Fits of the Bell model to the data are shown in corresponding colors. Black: a fit of the Bell model to the complete data set is shown.

measured force-dependent cis-FLNa20 unfolding rates were extrapolated to zero force k_{N_c, U_c}^0 using the Bell model (as explained in section 5.2). The resulting k_{N_c, U_c}^0 rate of $(1.9 \pm 0.1) \times 10^{-2} \text{ s}^{-1}$ and the $\Delta x = (12.8 \pm 0.1) \text{ nm}$ transition point distance are consistent with results from passive mode experiments.

9.2. Cis-FLNa20 Unfolding in the Presence of SlyD

In this section, cis-FLNa20 unfolding in the presence of SlyD is investigated. If SlyD binds stably to FLNa20, the resulting complex has a longer force-dependent lifetime compared to the corresponding native FLNa20 states.

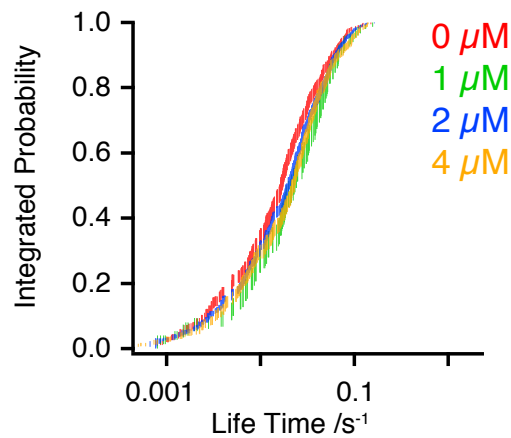


Figure 53 Integrated lifetime histograms for different SlyD concentrations: 0 μM shown in red, 1 μM in green, 2 μM in blue, and 4 μM in orange. Unfolding of N_c was observed at $(24.8 \pm 0.2) \text{ pN}$. *Jump experiment* parameters: $\tau_h = 5 \text{ ms}$, $\tau_l = 10 \text{ ms}$, $F_h = 24.8 \text{ pN}$ and $F_l = 1.1 \text{ pN}$

Figure 53 shows integrated N_c lifetime histograms under various SlyD con-

centrations. *Jump experiments* in the presence of SlyD were conducted to examine potential interactions between the native state of FLNa20 and SlyD. A SlyD bound N_c state is expected to be more stable and thus, the force-dependent lifetime longer than in the absence of SlyD. The N_c lifetimes were determined by applying HMM on the high force level un-/folding trajectories.

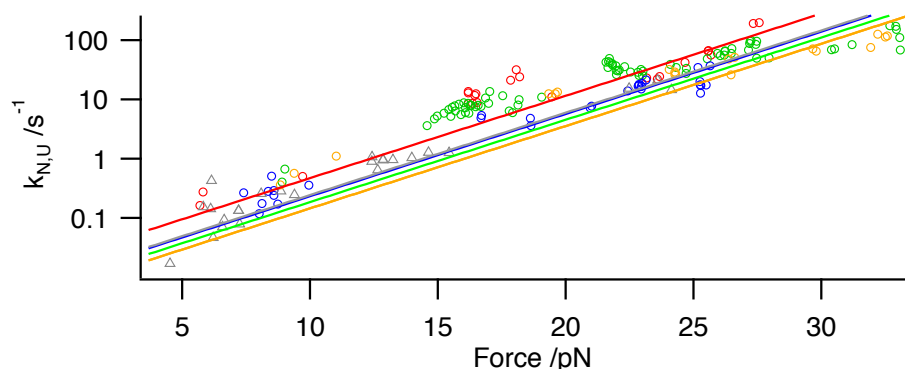


Figure 54 Unfolding rates of FLNa20 are shown in red and in presence of different SlyD concentrations, 1 μ M in green, 2 μ M in blue and 4 μ M in orange. Folding and unfolding fluctuations of FLNa20 under mechanical load has been measured with established methods [RMOR14]: gray triangles. Fits of the Bell-model to the data are extrapolated to zero force.

Figure 54 shows FLNa20 force-dependent unfolding rates in presence of up to 4 μ M SlyD. The affinity of SlyD binding to FLNa20 in the observed dumb-bell configuration is reported to be 3.3 μ M [Rog14]. Example un-/folding force trajectories in the presence of different SlyD concentrations are shown in figure 49. The lower force level for refolding was (1.0 ± 0.4) pN as discussed in detail later in section 9.3. Using the Bell model with $\Delta x = 12.8$ nm, the extrapolated unfolding rate k_{N_c, U_c}^0 of FLNa20 were found to be SlyD independent $(1.0 \pm 0.7) \times 10^{-3} \text{ s}^{-1}$.

9.3. Folding Kinetics of Filamin A 20

Protein folding is directly observable utilizing SMFS against a biasing force in the pN range and can then be extrapolated to zero force. In this section, cis-FLNa20 folding is measured under a negligible force via *jump experiments*. To accelerate un-/folding kinetics during a *jump experiment*, the biasing force level for refolding F_l is typically in a regime that is too low to resolve the contour length changes due to folding. From passive mode experiments, it is known, that the lifetime distribution of the unfolded peptide chain of the FLNa20 construct follows a single exponential distribution. Refolding rates under low biasing forces can be determined via *jump experiments* (section 7.5). The fraction of unfolded lifetimes that are longer than one jump dwell time at F_l was observed during *jump experiments*. Under the assumption that the unfolded lifetimes are single exponentially distributed, force dependent folding rates can be calculated using equa-

tion 7.25. Cis-FLNa20 folding kinetics can be observed performing a two-state *jump experiment* with F_h chosen such that the lifetimes of N_t are too short to be detected. All lifetimes of events with native state contour length can then be assigned N_c .

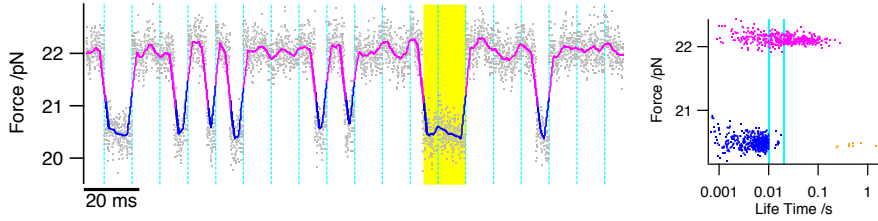


Figure 55 Stretch of an un-/folding force trajectory after post-processing (section 7.7.1), created from a *jump experiment* high force level. The vertical dotted cyan lines indicate the positions of jumps. The color of the smoothed data was assigned via HMM classifier; Pink: Cis folded, Blue: Cis unfolded, Orange: Trans unfolded. Proline isomerization phases (not shown in the trajectory) are separated using a time threshold. The vertical cyan lines indicate one and two jump dwell times at F_h . The yellow part of the trajectory highlights one unfolded lifetime that is longer than one jump period. Gray: Full bandwidth data. *Jump experiment* parameters: $\tau_h = 10$ ms, $\tau_l = 20$ ms, $F_h = 22$ pN and $F_l = 0.7$ pN

Post-processing, as explained in section 7.7.1, was applied to un-/folding force trajectories obtained during *jump experiments*. The merged high force level un-/folding force trajectories (figure 55) were then analyzed via HMM. Information about the conformational states at the low force level is hidden in this scenario. A lifetime threshold was used to separate U_c and U_t events. The vertical, cyan lines indicate one and two jump dwell times at F_h τ_h of 10 ms. Events shorter than these lines correspond to successful refoldings at the first or second attempt (section 7.7.3). Under a biasing force of 0.7 pN, 409 refoldings within a single jump dwell time of 20 ms were observed and 25 showed a longer lifetime. The probability of a first unsuccessful refolding attempt of U_c was 3.5 %. Under consideration of the missed events (equation 7.35), the folding rate of U_c under 0.7 pN was determined 142 s^{-1} by applying equation 7.34. No unfolded lifetimes longer than 2 jump cycles (40 ms in the right panel of figure 55) and shorter than 100 ms were observed. Longer unfolded lifetimes were assigned to trans phases.

Figure 56 shows, cis-FLNa20 folding rates $k_{U_c, N_c}(F)$ under a biasing force $F = (0.6 \pm 0.2)$ pN (in red) and folding rates determined from data measured during passive mode experiments with $F = 3 - 7$ pN (in gray). A fit of the *B-S model* (as described in section 5.3) to both datasets (black line in figure 56) was used to extrapolate the folding rate to zero force $k_{U_c, N_c}^0 = (185 \pm 40) \text{ s}^{-1}$ and determine the transition point distance $\Delta L = (16.2 \pm 0.2) \text{ nm}$ (black line in figure 56).

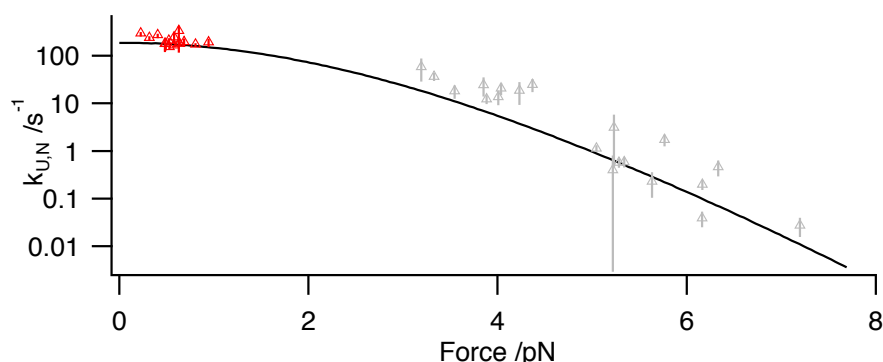


Figure 56 Force-dependent FLNa20 folding rates obtained during constant distance experiments: gray triangles. The folding rates measured during *jump experiments* are shown as red triangles. The black line indicates a fit of the *B-S model*.

9.4. Cis-FLNa20 Folding in the Presence of SlyD

The PPIase SlyD affects folding of certain proteins [ŽGS13]. In the present section, cis-FLNa20 folding rates in the presence of various SlyD concentrations are presented. A SlyD-dependent change of the FLNa20 folding kinetics would indicate an interaction of SlyD with the unfolded peptide chain. In a set of experiments, U_c folding rates in the presence of different concentrations of SlyD were measured.

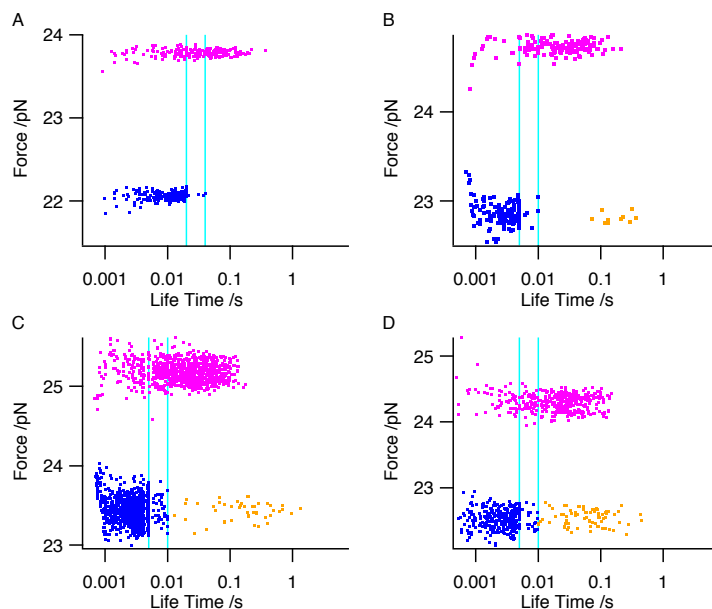


Figure 57 SlyD-dependent lifetimes of different conformational states of FLNa20:
A 0 M *jump experiment* parameters: $\tau_h = 20$ ms, $\tau_l = 20$ ms, $F_h = 23.6$ pN and $F_l = 0.6$ pN,
B 1 μ M *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 50$ ms, $F_h = 24.7$ pN and $F_l = 1.5$ pN,
C 2 μ M *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 20$ ms, $F_h = 25.2$ pN and $F_l = 0.6$ pN,
and D 4 μ M *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 20$ ms, $F_h = 24.3$ pN and $F_l = 0.7$ pN.

Figure 57 shows lifetimes of the conformational states determined by using the high force level un-/folding trajectory. Post-processing and data analysis was performed as explained above in section 7.7 (for example see figure 55). It is important to note, that the lifetimes do not represent the total lifetimes of the conformational states but they exist as well during the times spent at low force. The folding itself occurred at the low force level of (1.00 ± 0.45) pN. In the context of an unfolding study—unfortunately, in the presence of SlyD the lifetime of trans-FLNa20 decreases (see lifetime histograms in figure 53). When analyzing *jump experiments* as explained in chapter 7, one assumes that isomerization is slow compared to the jump frequency. The reported SlyD catalyzed proline isomerization rate is in the order of 100 s^{-1} [ŽGS13], thus with increasing SlyD concentration, the U_c and U_t lifetimes overlap (see bottom panels in figure 53). Nevertheless, the un-/folding kinetics can be determined even if the cis/trans assignment fails, by estimating the U_t lifetime distribution from long U_t lifetimes (see Appendix A.6).

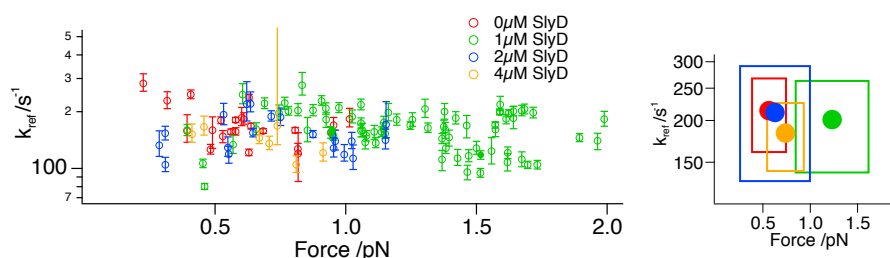


Figure 58 Force-dependent folding rates measured with *jump experiments* in the presence of different concentrations of SlyD. The individual values of various experiments are shown on the left. The mean and standard deviation for each concentration is shown on the right. The distributions overlap. Data for the different concentrations are shown in red: 0 μM , green: 1 μM , blue: 2 μM , and orange: 4 μM .

The measured force-dependent U_t folding rates are shown on the left panel in figure 58. The right panel shows the standard deviation for the different SlyD concentrations. Interestingly, the measured rates for the different SlyD concentrations appear fairly similar.

Under the assumption of no SlyD dependence, data obtained during *jump experiments* were pooled together. The pooled data were used together with data originating from constant distance experiments to specify force-dependent folding of FLNa20. Extrapolation of the constant distance data gave $K_{U,N}^0 = (410 \pm 60) \text{ s}^{-1}$ and $\Delta L = (18.4 \pm 0.6) \text{ nm}$, compared to $K_{U,N}^0 = (255 \pm 18) \text{ s}^{-1}$ and $\Delta L = (16.7 \pm 0.2) \text{ nm}$ obtained from the combined data set.

9.5. Discussion

It was reported, that proline isomerization limited protein folding is efficiently catalyzed by SlyD [ŽS11]. The PPIase SlyD has specific and effective en-

zymatic properties. Substrates are selected via high-affinity binding to the chaperone domain. In contrast, the prolyl isomerase site of SlyD, located in the FKBP-domain, features very low substrate affinity and high turnover rate. The effective, selective isomerization is ensured by the substrate-specific high binding affinity of the chaperone domain. Protein molecules that left the prolyl isomerase site with an incorrect prolyl isomer can immediately be rebound to the chaperone site because the association rate is very high. If the chaperone domain is capable of binding native folded substrates, this effective catalyzed isomerization mechanism applies as well to proteins with different native states, and functions for different proline isomerization states. Constant distance experiments in the presence of SlyD are not capable of discriminating N_t against N_c . A state where SlyD is bound to N_c is expected to elongate its lifetime.

Unfolding rates obtained during a series of *jump experiments* covering a wide range of jump-parameters were compared with results measured in constant distance experiments. Extrapolated to zero force, the measured N_c unfolding rate was $k_{N,U}^0 = 9.3\text{--}17.4\text{ s}^{-1}$. This excellent agreement between the different experimental protocols is even more remarkable, considering that the experiments were conducted over several years, using different sample preparations and multiple optical tweezers instruments. It was reported, that folded or highly charged proteins or peptides barely interact with SlyD [WHS⁺09]. If FLNa20 in the N_c conformation interacts with SlyD, stabilization of the N_c state, and thus longer lifetimes are expected. FLNa20 unfolding rates in presence of up to 4 μM SlyD were successfully measured. The measured data showed no trend that would indicate a SlyD dependence of N_c unfolding (figure 54). The variation in extrapolated zero force unfolding rates $5.9\text{--}17.4\text{ s}^{-1}$ was addressed to experimental noise. The results of the *jump experiments* indicate that there was no folding incompetent SlyD bound N_c state.

Folding of U_t was not directly observable during *jump experiments*. Resolution of the contour length change due to the folding of FLNa20 is restricted to biasing forces that slow down folding. Usually, during a *jump experiment*, the direct observation of folding is traded for faster folding kinetics. In all *jump experiments* that are presented in this work, folding was not resolved. Widely used SMFS protocols (constant velocity cycles or passive mode experiments) are not capable of observing folding without a biasing force. The *B-S model* can be used to extrapolate folding rates to zero force conditions. In section 7.7.3 a method is introduced to estimate the folding rate under neglectable force bias in fast *jump experiments*. The folding results obtained during *jump experiments* showed good agreement with results from passive mode SMFS experiments. Force-dependent folding is described by the transition rate under zero force and the relative position of the transition state Δx (section 5.2). During *jump experiments*, folding was observed under a force in a narrow low regime under which unfolding is improbable. Variations in the measured force and folding rate was considered ex-

perimental noise, thus Δx was not determined by *jump experiments*. The data presented in figure 56 is a direct experimental confirmation of the *B-S model*. The results confirm both, the zero force extrapolation via the *B-S model* as well as the kinetic rates determined using *jump experiments*.

Various studies reported SlyD capable of stably binding unfolded peptide chains e.g. [WHS⁺09], [ŽGS13], [KZH⁺11], [LNT⁺10], [KES⁺07]. The SlyD bound state of the sample peptide with a lifetime of 25 ms was reported to be folding incompetent. During the *jump experiments* presented here, no change in FLNa20 un-/folding kinetics was found in the presence of SlyD.

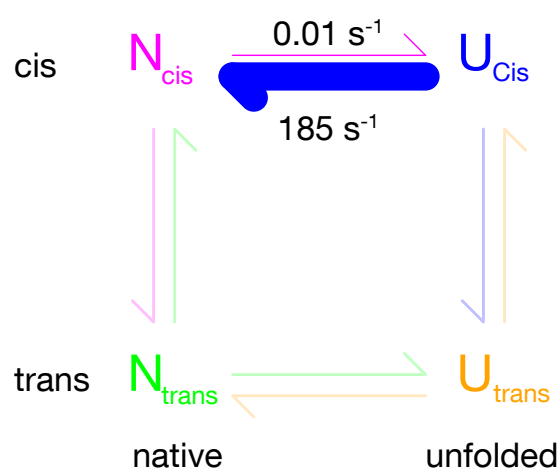


Figure 59 The kinetic network of FLNa20 states. Un-/folding is not affected by the presence of SlyD. In the trans-state, un-/folding transitions are not observed during typical *jump experiments*.

9.6. Summary

The new fast-force-jump protocol was confirmed to control the un-/folding kinetics of proteins, needed for SMFS on complex kinetic networks. The probe in such studies is the conformational states and the transition kinetics of the sample protein, thus its un-/folding kinetics limit the temporal resolution. Using the *jump experiment* protocol, the temporal resolution is the zero force folding time or the one millisecond response time of the dumbbell, whichever is slower. Folding rates measured with *jump experiments* were in accord with experimental data obtained during passive mode experiments (figure 56). In conclusion, no SlyD dependence of the FLNa20 lifetimes was observed during *jump experiments*.

10. Isomerization Kinetics of FLNa20

In the following, a thorough investigation of proline isomerization kinetics in FLNa20 under various SlyD concentrations is presented. A detailed kinetic description of proline isomerization in FLNa20 with and without the enzyme SlyD unveils the underlying molecular mechanism. The kinetic network of FLNa20 consists of four conformational states, N_c , N_t , U_c , and U_t . During passive mode experiments, the cis- and trans-states were observed by their different lifetimes under force. The isomerization rates were determined via the lifetimes of the cis and trans-states. The cis and trans lifetimes were measured by observing consecutive un-/folding events. The isomerization kinetics of FLNa20 under force has been measured by passive mode SMFS [RMOR14]. The experiments require folding kinetics being significantly faster than the proline isomerization kinetics. Members of the enzyme family of PPLases catalyze proline isomerization. In the presence of SlyD, enzymatic proline isomerization occurs on timescales shorter than equilibrium un-/folding kinetics in passive mode experiments. Back and forth proline isomerization can take place within a single unfolded lifetime, thus, a non-negligible fraction of proline isomerization events are missed.

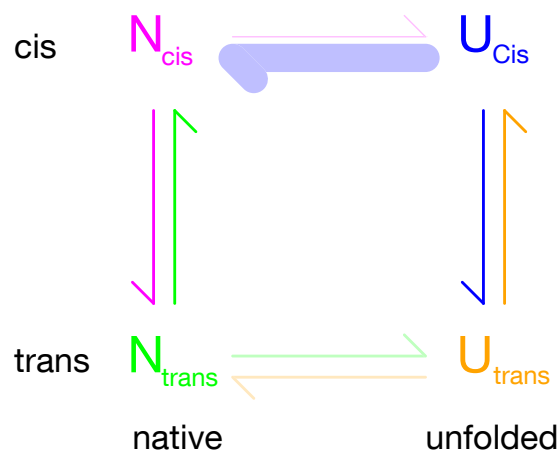


Figure 60 Kinetic network of FLNa20 states. The proline isomerization transitions are shown in bright colors.

Figure 60 shows the proline isomerization transitions in the kinetic network of FLNa20 in bright colors. During the *jump experiments* presented in chapter 9 there was no signal of SlyD binding to FLNa20, thus the non-enzymatic and enzymatic (shown in identical colors in figure 60) isomerization paths are not distinguishable. The fast-force-jump protocol was developed to study complex kinetic networks that contain proline isomerization. Un-/folding trajectories obtained during *jump experiments* in the presence

of different SlyD concentrations showed different un-/folding behavior (figure 49). The apparent isomerization rates $k_{c,t}^{\text{app}}$ and $k_{t,c}^{\text{app}}$ (defined by equation 7.26) were calculated via the measured lifetimes of the cis and trans-state (as explained in section 7.6).

10.1. Unfolded *Cis-Trans* Isomerization

In a first set of *jump experiments*, force-dependent FLNa20 *cis-trans*-isomerization rates were measured. Non-enzymatic FLNa20 *cis-trans*-isomerization in the unfolded state was reported to be multiple orders of magnitude faster than in the native state [RMOR14]. The state population probabilities during a *jump experiment* were determined via the un-/folding rates (as measured in chapter 9) and the jump dwell times (as experimental parameters).

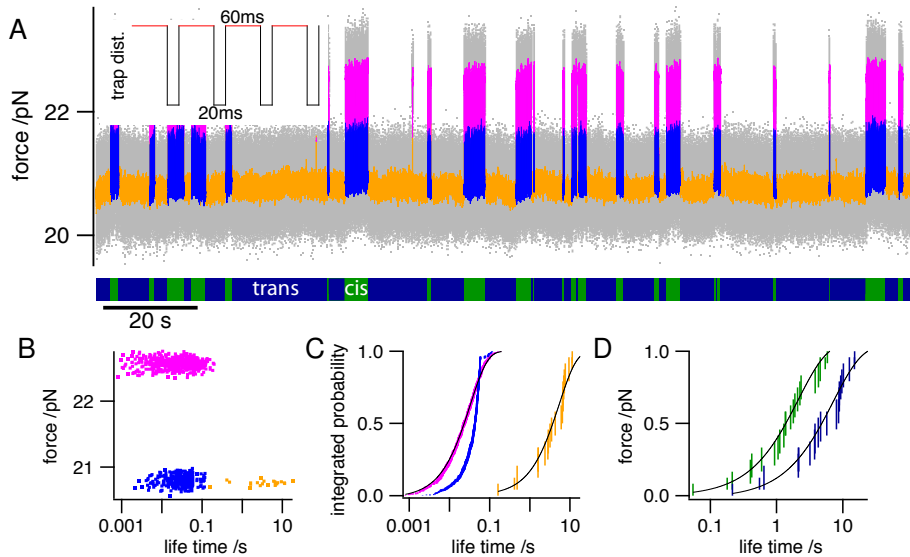


Figure 61 Un-/folding force trajectory created from a *jump experiment* high force level (red parts (A) insert), in presence of 1 μM SlyD (A). *Jump experiment* parameters: $\tau_h = 60$ ms, $\tau_l = 20$ ms, $F_h = 22.5$ pN and $F_l = 0.9$ pN. The folded — N_c — and unfolded FLNa20 states were assigned via HMM. The U_c and U_t lifetimes were separated by application of a threshold on the unfolded lifetimes (C). Full bandwidth data is shown in gray, the smoothed trajectory colored in pink indicates N_c , in blue U_c and in orange U_t . Times assigned to U_t are trans (blue bars), other are cis (green bars). The integrated lifetime histograms of the conformational states were created directly from the obtained lifetimes (C). To create cumulative histograms of the isomerization states the lifetimes were multiplied by the jump dwell times factor $\frac{\tau_h}{\tau_l}$ (D).

After data preparation (section 7.7.1) and determination of the un-/folding rates (section 7.7.3 and section 7.5), isomerization analysis was possible. Figure 61A shows a typical un-/folding force trajectory in presence of 1 μM SlyD. Cis and trans phases were assigned using a threshold on the unfolded lifetimes (figure 61B). The apparent *cis-trans*-isomerization rate was determined via cis lifetimes and yielded $k_{c,t}^{\text{app}} = \frac{n}{\sum \tau_c} = 0.47 \text{ s}^{-1}$. The population probabilities during a *jump experiment* with the following parameters:

$F_l = 0.9$ pN, $F_h = 22.5$ pN, $\tau_s = 20$ ms and $\tau_h = 60$ ms was determined applying equation 7.30 $p_{U_c}^l = 6.6\%$ and $p_{U_c}^h = 40.6\%$. The force-dependent unfolded *cis-trans*-isomerization rate was determined according to equation 7.27: under low force $k_{U_c, U_t}(0.9 \text{ pN}) = (0.010 \pm 0.002) \text{ s}^{-1}$ and under high force $k_{U_c, U_t}(20.8 \text{ pN}) = (1.15 \pm 0.23) \text{ s}^{-1}$. In total 24 proline isomerizations events were observed in the example presented in figure 61, thus the statistical error $\frac{1}{\sqrt{n}}$ was 20 %.

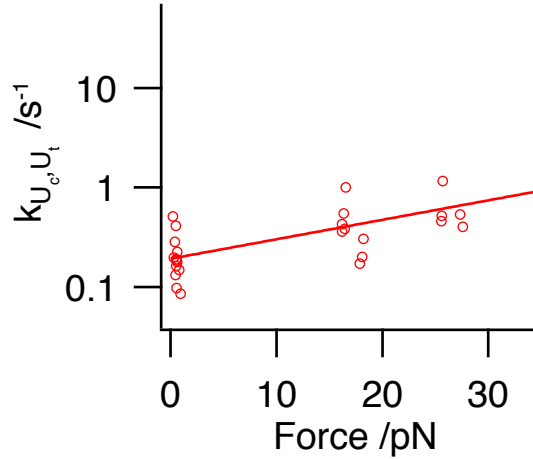


Figure 62 Force-dependent FLNa20 *cis-trans*-Isomerization rates measured during *jump experiments*. The solid line indicates a fit of the Bell model to the data.

Figure 62 shows force-dependent *cis-trans*-isomerization under different SlyD concentrations. Proline isomerization is an intrinsically slow process and for high resolution during a *jump experiment*, *cis* and *trans*-lifetimes of multiple jump cycles are desirable. Thus, a limited number of isomerization events (typically > 20) were taken into account for each data point. The force-dependent *cis-trans*-isomerization was extrapolated to zero force via a fit of the *B-S model* to the data. Using a transition point distance of $\Delta x = 0.17$ nm, k_{U_c, U_t}^0 yielded $(0.19 \pm 0.03) \text{ s}^{-1}$.

10.2. Enzymatic, Unfolded *Cis-Trans* Isomerization

In the present section, unfolded FLNa20 *cis-trans*-isomerization rates under different SlyD concentrations are investigated. For SlyD concentrations higher than $2 \mu\text{M}$ the *trans* lifetimes were comparable to the U_c lifetime, thus assignment of *cis* and *trans* phases is challenging. Data presented here were obtained during *jump experiments* in presence of up to $2 \mu\text{M}$ SlyD. The extracted unfolded *cis-trans*-isomerization rates under zero force, for different SlyD concentrations $k_{U_c, U_t}^0(c_{\text{SlyD}})$ showed an enzymatic activity. Due to the Δx value of 0.17 nm (Appendix C.2) and the resulting weak force dependence, binning of the data is suitable for visualization.

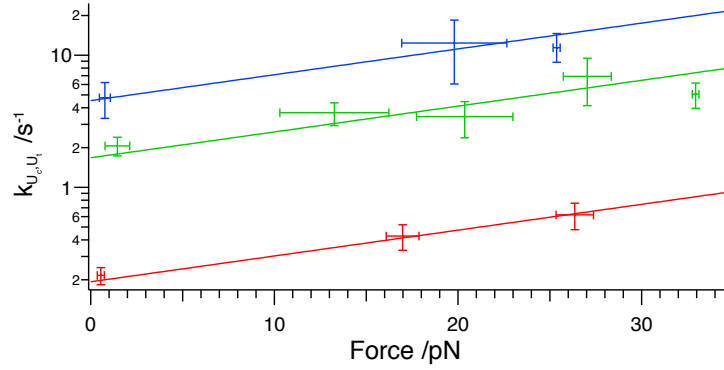


Figure 63 Force-dependent *cis-trans*-isomerization of FLNa20 in its unfolded state. Binned isomerization data and fits of the Bell model to the unbinned data (solid lines) are shown. Binned data for zero SlyD (red), 1 μM (green) and 2 μM (blue).

Figure 63 shows force-dependent *cis-trans*-isomerization rates in presence of SlyD, divided into force bins with 5 pN. The force-dependent *cis-trans*-isomerization were extrapolated to zero force via a fit of the *B-S model* to the data. The *cis-trans*-isomerization rate was $k_{U_c, U_t}^0 = (1.7 \pm 0.3) \text{ s}^{-1}$ in the presence of 1 μM and $(4.5 \pm 1.4) \text{ s}^{-1}$ under 2 μM SlyD, compared to $(0.19 \pm 0.03) \text{ s}^{-1}$ measured in absence of PPlase.

10.3. Unfolded *Trans-Cis* Isomerization

The results of the FLNa20 *trans-cis*-isomerization study are presented in the present section. In contrast to *cis-trans*-isomerization, native state *trans-cis*-isomerization contributes non-negligible to $k_{t,c}^{\text{app}}$ during *jump experiments*. In addition, U_t folding is not observable, and thus the population probabilities remain hidden. Data obtained during a single *jump experiment* is not suitable to measure the force-dependent unfolded *trans-cis*-isomerization rate $k_{U_t, U_c}(F)$. Simulations have shown, that k_{U_t, U_c} can be extracted from two *jump experiments* protocol, using different high force jump dwell time τ_h (equation 7.36).

As figure 43 shows, the apparent *trans-cis*-isomerization rate $k_{t,c}^{\text{app}}$ in presence of 1 μM SlyD, depends on F_h jump dwell time τ_h . In both *jump experiments*, the jump dwell time under low force was $\tau_l = 20 \text{ ms}$ and the high force level $F_h \approx 22 \text{ pN}$. With the observed $k_{t,c}^{\text{app}} = (0.33 \pm 0.10) \text{ s}^{-1}$ at $\tau_h = 10 \text{ ms}$ and $k_{t,c}^{\text{app}} = (0.16 \pm 0.04) \text{ s}^{-1}$ at $\tau_h = 60 \text{ ms}$, the unfolded *trans-cis*-isomerization rate was calculated using equation 7.36: $k_{U_t, U_c}(20.5 \text{ pN}) = (0.06 \pm 0.05) \text{ s}^{-1}$. Unfolded FLNa20 *trans-cis*-isomerization was measured either in the absence or in presence of 1 μM SlyD. Since force dependent unfolded *trans-cis*-isomerization rate is measured via two *jump experiments*, stable experimental conditions are needed. The k_{U_t, U_c} uncertainty was calculated using equation 7.37 might be bigger than the individual ap-

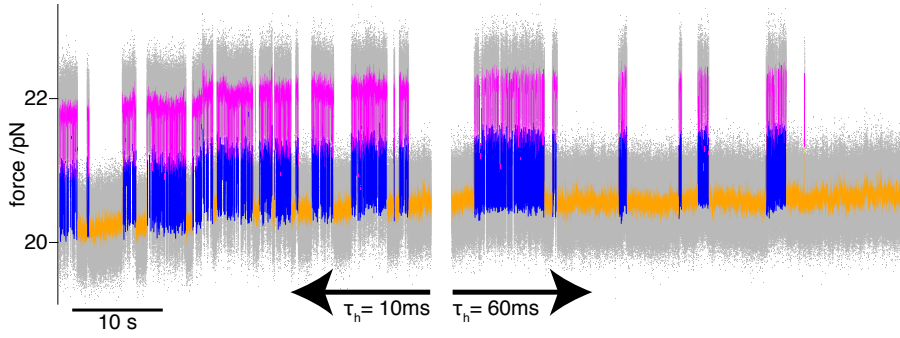


Figure 64 FLNa20 un-/folding force trajectories obtained during *jump experiments* with different τ_h after data preparation (as explained in section 7.7.1). The colors are assigned using an HMM N_c in pink, U_c in blue and U_t in orange. The apparent cis and trans isomerization behavior is different with *jump experiment* parameters: $\tau_h = 10$ ms, $\tau_l = 20$ ms, $F_h = 22.0$ pN and $F_l = 0.7$ pN (left) compared to *jump experiment* parameters: $\tau_h = 60$ ms, $\tau_l = 20$ ms, $F_h = 22.2$ pN and $F_l = 0.8$ pN (right).

parent rate's $k_{t,c}^{\text{app}}$ errors.

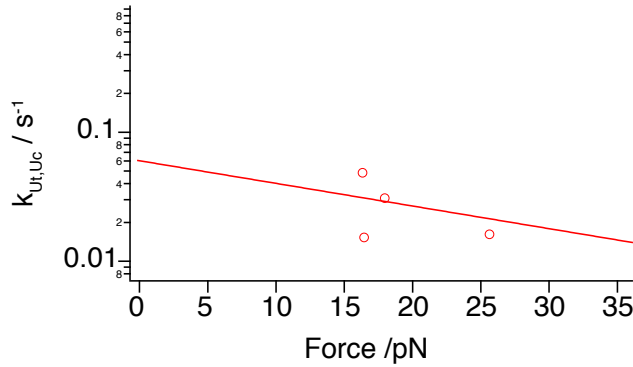


Figure 65 Unfolded *trans-cis* isomerization in the absence of SlyD obtained during *jump experiments*. The solid line represents a fit of the Bell model to the data.

Figure 65 shows results obtained during *jump experiments*. The *trans-cis*-isomerization rate extrapolated to zero force is a fit of the Bell model to the data. A transition point distance of $\Delta x = 0.17$ nm as reported in Ref. [RMOR14] was used to extrapolate the force-dependent *trans-cis*-isomerization rates to zero force (as in section 10.1). The unfolded *trans-cis*-isomerization rate was $k_{U_t,U_c}^0 = (0.06 \pm 0.01) \text{ s}^{-1}$.

10.4. Enzymatic Unfolded *Trans-Cis* Isomerization

The force-dependent FLNa20 *trans-cis*-isomerization rates under various SlyD concentrations are subject of the present section. The *jump experiments* to determine the force-dependent rates are described in section 10.3.

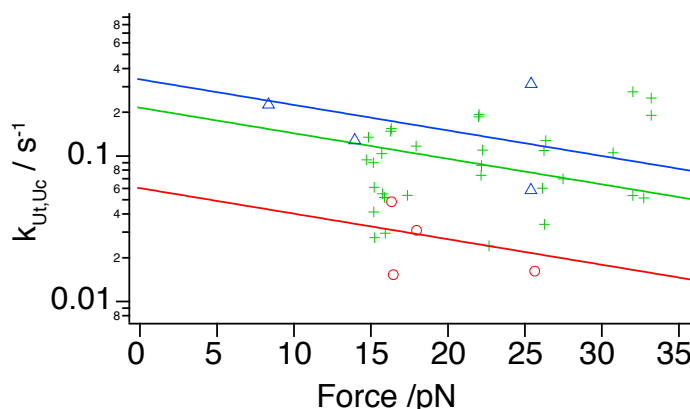


Figure 66 Force-dependent *trans-cis*-isomerization rates of unfolded FLNa20 in the presence of 1 μM as green crosses, 2 μM as blue triangles and without SlyD as red circles. Corresponding fits of the Bell model to the *trans-cis*-isomerization rates are shown as solid lines.

Figure 66 shows the force-dependent *trans-cis*-isomerization rates in presence of different SlyD concentrations, together with corresponding fits of the Bell model. The transition point distance $\Delta x = 0.17$ nm was used to determine the zero force *trans-cis*-isomerization rate $k_{\text{U},\text{Uc}}^0$. In the presence of 1 μM SlyD the *trans-cis*-isomerization rate $k_{\text{U},\text{Uc}}^0$ was $(0.21 \pm 0.03) \text{ s}^{-1}$ and $k_{\text{U},\text{Uc}}^0 = (0.34 \pm 0.12) \text{ s}^{-1}$ under 2 μM SlyD.

10.5. Enzymatic Native State *Trans-Cis* Isomerization

A main goal of this work was to unveil whether FLNa20 native state proline isomerization is catalyzed by the PPlase SlyD. In the present section, results of *jump experiments* to measure native state *trans-cis*-isomerization $k_{\text{Nt},\text{Nc}}$ in the presence of different SlyD concentrations are presented. During *jump experiments* simulations, the τ_h -dependent apparent *trans-cis*-isomerization rate is related to the native state *trans-cis*-isomerization rate (section 10). If $k_{\text{t},\text{c}}^{\text{app}}$ was considerably higher for short τ_h than for long, if $k_{\text{Nt},\text{Nc}}$ is larger than $k_{\text{U},\text{Uc}}^0$. In a scenario of slow $k_{\text{Nt},\text{Nc}}$, the $k_{\text{t},\text{c}}^{\text{app}}$ against τ_h diagram is flat.

Apparent *trans-cis*-isomerization rates $k_{\text{t},\text{c}}^{\text{app}}(\tau_h)$ obtained during different *jump experiments* are shown in figure 67. Depending on the experimental parameter of the *jump experiment* the apparent *trans-cis*-isomerization rate $k_{\text{t},\text{c}}^{\text{app}}$ is higher or lower. For *jump experiments* with similar parameters but different high force jump dwell time, the rates are higher for shorter τ_h .

Kinetic simulations (as explained in section 7.2) were used for forward modeling the *jump experiment* series (figure 68). The *jump experiments* were carried out at a high force $F_h = 27$ pN, waiting 10 ms at 0.6 pN in presence

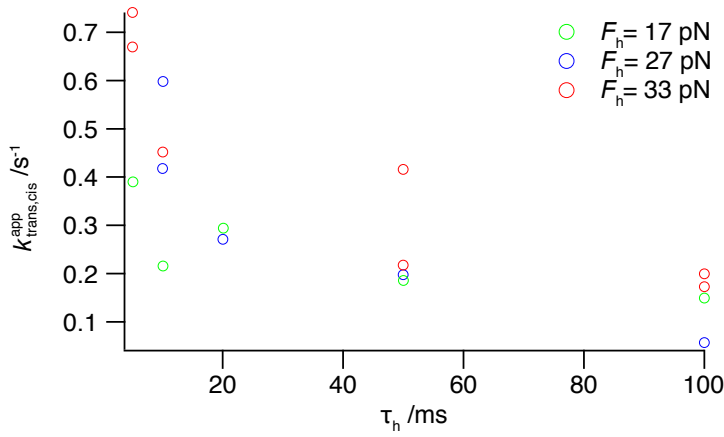


Figure 67 Apparent *trans-cis*-isomerization rates over τ_h , obtained during *jump experiments* with different high force levels. *Jump experiment* parameters $\tau_l = 10$ ms, $F_l = 1.5$ pN. The absolute $k_{t,c}^{\text{app}}$ values depend on the experimental parameters. Among data obtained using similar jumping parameters $k_{t,c}^{\text{app}}$ is higher for short τ_h .

of 1 μM SlyD. Simulations using the non-enzymatic native state isomerization rate k_{N_t, N_c} as input were below the experimental data. Data obtained during *jump experiments* are within simulations using $k_{N_t, N_c} = 1 - 3 \text{ s}^{-1}$. Ten-fold increased native state *cis-trans*-isomerization does not influence the apparent rate $k_{c,t}^{\text{app}}$.

If the force-dependent *trans-cis*-isomerization rate $k_{U_t, U_c}(F)$ is known, the native state rate k_{N_t, N_c} can be calculated using equation 7.26. The native state population probability is calculated from the trans-FLNa20 folding rate $k_{U_t, N_t} = (89 \pm 2) \text{ s}^{-1}$ (chapter 11) and the low force jump dwell time τ_l (using equation 7.5 and equation 7.7).

The k_{N_t, N_c} histograms shown in figure 69, are shifted in the presence of SlyD. The measured *trans-cis*-isomerization rates $k_{N_t, N_c} < 0.23 \text{ s}^{-1}$ in absence of PPlase and $k_{N_t, N_c} = (0.98 \pm 0.65) \text{ s}^{-1}$ in presence of 1 μM SlyD were consistent with the results determined via forward modeling.

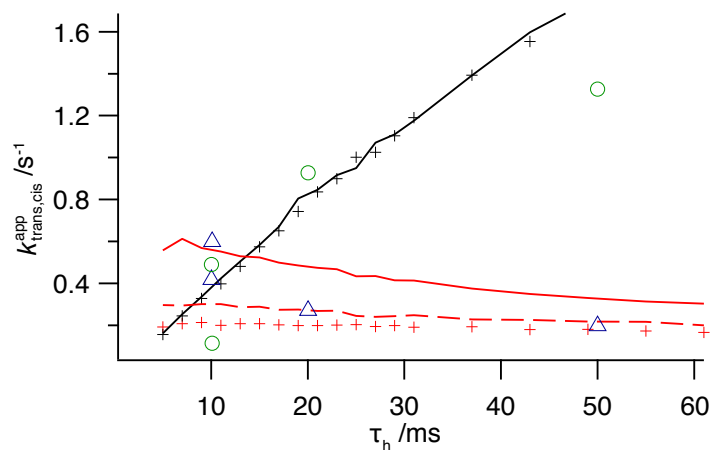


Figure 68 Apparent isomerization rates $k_{t,c}^{\text{app}}$ (as blue triangles) and $k_{c,t}^{\text{app}}$ (as green circles) obtained during *jump experiments* with similar parameter under $1 \mu\text{M}$ SlyD. *Jump experiment* parameters $\tau_1 = 10 \text{ ms}$, $F_h = 27 \text{ pN}$, $F_l = 1.5 \text{ pN}$. Simulations using non-enzymatic native state and the measured unfolded isomerization rates (*cis-trans*-isomerization as black crosses, *trans-cis*-isomerization as red crosses). Solid lines are simulations with increased native state isomerization rate (tenfold increased *cis-trans*-isomerization in black and 3 s^{-1} *trans-cis*-isomerization in red). Broken red line, native state *trans-cis*-isomerization rate $k_{Nt,Nc} = 1 \text{ s}^{-1}$.

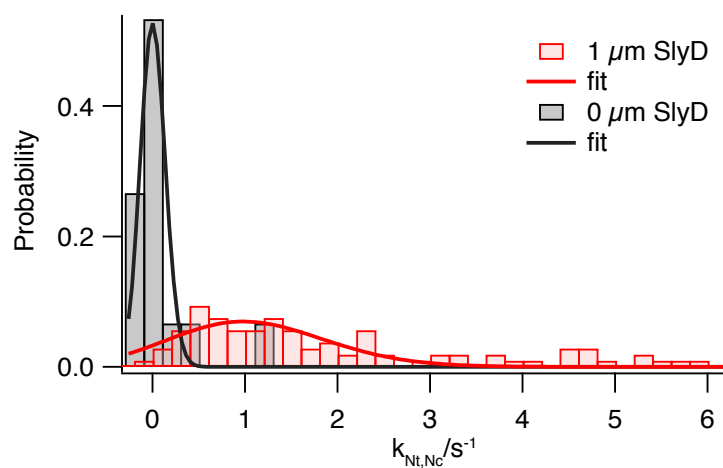


Figure 69 Native state *trans-cis*-isomerization rates $k_{Nt,Nc}$ obtained during *jump experiments*. Non-enzymatic rates are shown as black bars and in the presence of $1 \mu\text{M}$ SlyD as red bars. Solid lines are fits of the *Log-normal-distribution* to the data.

10.6. Enzymatic Native State *Cis-Trans* Isomerization

Jump experiments that are appropriate to observe FLNa20 native state *cis-trans*-isomerization and to check if it is catalyzed by SlyD are subject of the present section. In FLNa20's kinetic network, k_{N_c, N_t} is the transition that occurs at the smallest rate, considered throughout this work. N_c unfolds at a rate of $k_{N_c, U_c}^0 = 0.019 \text{ s}^{-1}$. Isomerization of *cis* p2225 in N_c to N_t is a rare event with a rate of $5.5 \times 10^{-5} \text{ s}^{-1}$ [RMOR14]. To measure k_{N_c, N_t} , it is important to suppress the dominant *pathway*: FLNa20 unfolding first, followed by *trans-cis*-isomerization. During *jump experiments* simulations with $F_h < 10 \text{ pN}$, k_{N_c, N_t} is observable (figure 45).

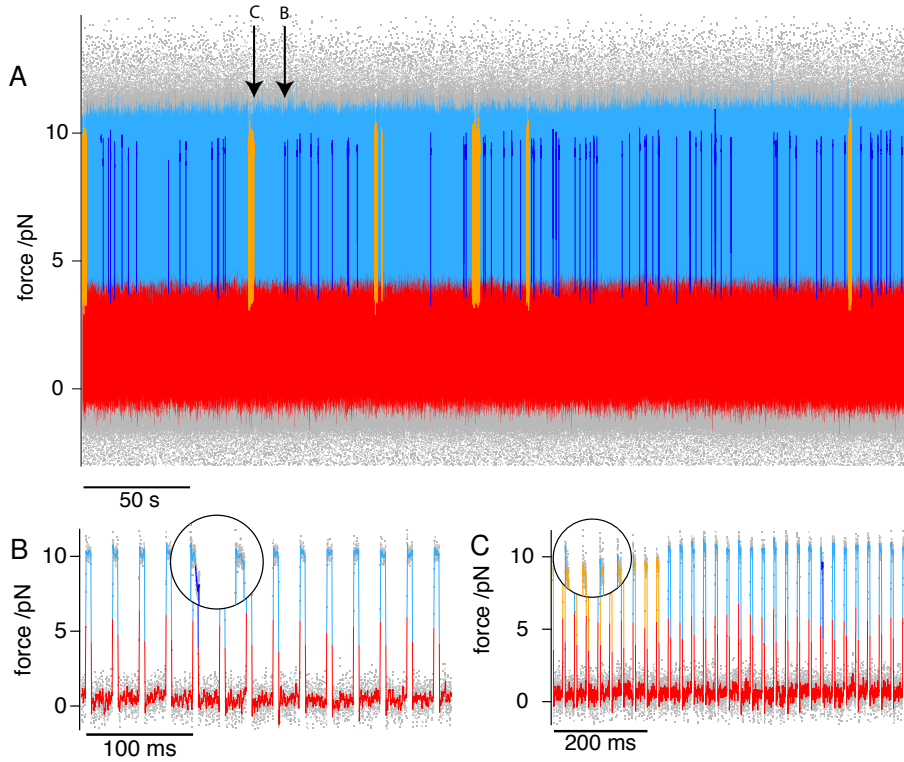


Figure 70 FLNa20 force trajectory obtained during a *jump experiment* to F_h 10 pN. *Jump experiment* parameters: $\tau_h = 5 \text{ ms}$, $\tau_l = 20 \text{ ms}$, $F_h = 10.4 \text{ pN}$ and $F_l = 0.5 \text{ pN}$. The native (bright blue) and unfolded state (dark blue and orange) at F_h , and the F_l force level (red) are assigned via HMM. Jump dwell times τ_h 5 ms and τ_l 20 ms allow fast U_c refolding. N_c unfolding at $F_h = 10 \text{ pN}$ is slow. Thus *cis*-FLNa20 phases are identified by long native lifetimes with occasional short unfolded events (B). Single unfolding events are assigned U_c (dark blue) and subsequent un-/folding transitions between U_t (orange) and N_t .

Jump experiments with $F_h \leq 10 \text{ pN}$ were performed to observe the rare native state *cis-trans*-isomerization. Under such conditions, N_c is dominantly populated $p(N_c) \geq 0.998$ (figure 70). In contrast to the data that are shown above, FLNa20's native *trans*-state is detectable under a biasing force $F \leq 10 \text{ pN}$ (highlight in figure 70C). Phases of rapid fluctuation between the native and the unfolded state are assigned *trans*-FLNa20 (left

part in figure 70C). In the *cis*-conformation, FLNa20 un-/folding fluctuations occur as isolated events (highlight in figure 70B).

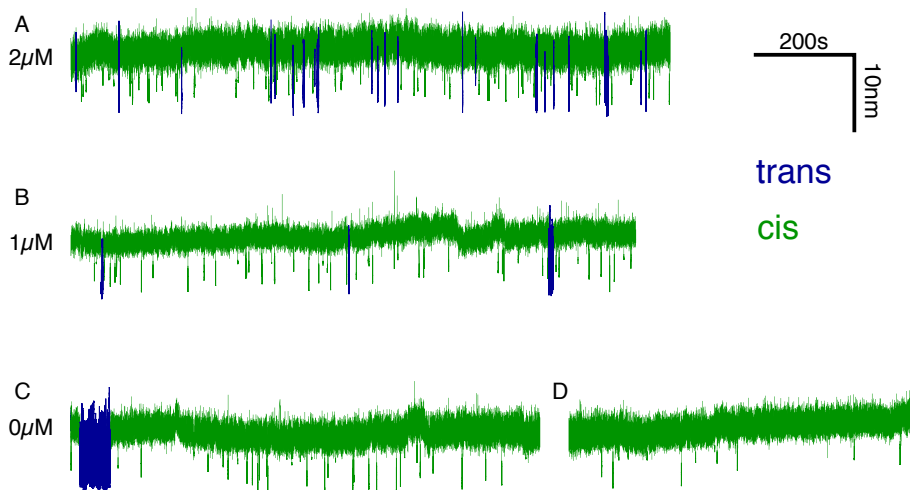


Figure 71 FLNa20 time traces obtained during *jump experiment* after post-processing (as explained in section 7.7.1). The force trajectories show 2 different levels, corresponding to the dominantly populated native and the unfolded state. FLNa20 being in its *cis*-state is colored in green, the *trans* in blue. A *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 20$ ms, $F_h = 7.5$ pN and $F_l = 0.3$ pN 2 μM SlyD, B *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 40$ ms, $F_h = 8.9$ pN and $F_l = 1.4$ pN, 1 μM SlyD, C *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 20$ ms, $F_h = 5.8$ pN and $F_l = 0.4$ pN, 0 M SlyD, D *jump experiment* parameters: $\tau_h = 5$ ms, $\tau_l = 20$ ms, $F_h = 5.7$ pN and $F_l = 0.8$ pN, 0 M SlyD

FLNa20 *cis* and *trans* lifetimes were measured in presence of up to 2 μM SlyD. Figure 71 shows un-/folding force trajectories after post-processing (as explained in section 7.7.1) with *cis* and *trans* phases assigned. The proline isomerization kinetics were faster for higher SlyD concentrations. Population probabilities $p(N_c)$, $p(U_c)$ as well as the *cis* lifetimes τ_c were determined. Native state *cis-trans*-isomerization rates k_{N_c, N_t} can be determined from simulated *jump experiments* with equivalent parameters (figure 78).

The *cis-trans*-isomerization rates $k_{c,t}^{app}$ observed during these *jump experiments* are slow, in particular in absence of SlyD. Apparent isomerization rates $k_{c,t}^{app}$ observed during *jump experiment* are sensitive to experimental parameters e.g. F_h , thus direct comparison of $k_{c,t}^{app}$ from different experiments is not appropriate. It is possible to pool *cis* lifetimes from different experiments (see Appendix A.5), but due to non-enzymatic *cis* lifetimes of 10^5 s it is experimentally challenging to gather a sufficient amount of data.

Due to the limited amount of experimental data that is available, the native state *cis-trans*-isomerization rates were not directly measured. The native state *cis-trans*-isomerization rate k_{N_c, N_t} was determined as an inverse problem, using kinetic simulations (as explained in section 7.2) with experimentally observed $k_{c,t}^{app}$ and measured k_{U_c, U_t} as input. A corresponding number of *cis-trans*-isomerizations with equivalent jump parameters were simulated such that the results gave the same $k_{c,t}^{app}$ as the experiment. The solutions for the hypothetical scenarios of k_{U_c, U_t} measured with jump exper-

Table 2 FLNa20 *cis-trans*-isomerization rates $k_{c,t}^{\text{app}}$ under *jump experiment* conditions. The experimental data (see: figure 71) and forward modeling using two different scenarios: one using SlyD catalyzed k_{U_c,U_t} as measured before (table 2) assuming only catalysis of k_{U_c,U_t} . The simulations one σ confidence intervals are given behind the $k_{c,t}^{\text{app}}$ values (the intervals have been determined by 1000 simulations).

	$k_{U_c,U_t}^0 / \text{s}^{-1}$	$k_{N_c,N_t} / \text{s}^{-1}$	$k_{c,t}^{\text{app}} / \text{s}^{-1}$
Exp. $c(\text{SlyD}) = 2 \mu\text{M}$			0.022 ± 0.005
Simulation	4.5	0.0212	0.021 [0.018, 0.028]
Simulation	147	5.5×10^{-5}	0.019 [0.016, 0.029]
Exp. $c(\text{SlyD}) = 1 \mu\text{M}$			0.002 ± 0.00024
Simulation	1.7	0.003	0.0021 [0.0021, 0.0075]
Simulation	20	5.5×10^{-5}	0.0023 [0.0019, 0.0070]

iments (as determined in section 10.2) and non-enzymatic k_{N_c,N_t} are shown in table 2.

10.7. Discussion

Local atomic displacements in proteins due to proline isomerization are $\approx 1 \text{ \AA}$ and propagation through the protein is unlikely [RF02]. In FLNa20, two distinct native states originating in proline isomerization act as a force sensor [RMOR14]. Notably, during SMFS experiments no contour length change is detectable due to p2225 undergoing proline isomerization. The proline isomerization-induced bimodal stability of the native FLNa20 state was exploited to measure transition rates in the kinetic network of states, using passive mode SMFS [RMOR14]. The PPlase SlyD interacts with FLNa20, increasing the kinetic network's intricacy [ŽS11]. However, passive mode SMFS is not appropriate to study enzymatic proline isomerization kinetics faster than force biased FLNa20 folding rates of 1 s^{-1} . Kinetic simulations showed that *jump experiments* are sensitive to all individual transition rates in FLNa20's complex kinetic network of states in presence of SlyD (chapter 10).

FLNa20 populates four distinct states forming a kinetic network of native and unfolded states with the p2225 being either in *trans*- or *cis*-conformation. SlyD accelerates the proline isomerization between corresponding *cis* and *trans*-states [ŽGS13]. A proposed mechanism of the enzymatic proline isomerization consists of two consecutive steps [Rog14]. First binding of the chaperone domain of SlyD to the unfolded peptide chain occurs, as reported before [SEH⁺06], [ŽS11]. In the bound state, the FKPF domain is in

close proximity of the proline, catalyzing its isomerization. While bound the peptide chain is folding incompetent and the proline undergoes fast back and forth proline isomerization transitions [ŽGS13].

During *jump experiments*, the unfolded state proline isomerization rates were 5–10 fold increased in presence of 1 μM SlyD. Furthermore, native state *trans-cis*-isomerization rates were found considerable increased. In the native state, the *trans-cis*-isomerization rate was measured via two *jump experiments* that differ in τ_h . The non-enzymatic *trans-cis*-isomerization rate was determined $< 0.23 \text{ s}^{-1}$, in agreement with previously determined via passive mode SMFS 0.046 s^{-1} [RMOR14]. In the presence of 1 μM SlyD, the *trans-cis*-isomerization rate yielded 0.98 s^{-1} , that corresponds to a twenty-fold increased *trans-cis*-isomerization rate with respect to the passive mode experiments presented in Ref. [RMOR14].

Due to the intrinsic slow native state *cis-trans*-isomerization, it is experimentally challenging to gather a sufficient amount of data for direct k_{N_c, N_t} determination. Concerning the limited number of observed *cis-trans*-isomerization during low F_h *jump experiments*, the native state *cis-trans*-isomerization rate k_{N_c, N_t} was determined via an inverse problem. Thus, kinetic simulations were used to determine k_{N_c, N_t} . Using the experimentally measured force-dependent unfolded *cis-trans*-isomerization rate $k_{U_c, U_t}(F)$ as simulation input, SlyD was found to increase native state *cis-trans*-isomerization rate. Keeping the non-enzymatic native state *cis-trans*-isomerization rate as simulation input, the consistent unfolded state enzymatic *cis-trans*-isomerization $k_{U_c, U_t}(F)$ was 10 fold higher than measured during *jump experiments* with $F_h = 15 - 25 \text{ pN}$. However, neither passive mode SMFS [Rog14], nor molecular dynamic simulations [CEGB12] support such a *cis-trans*-isomerization force dependence. In the presence of 1 μM SlyD, the native state *cis-trans*-isomerization rate was found to be twenty fold increased and considerably faster in the presence of 2 μM SlyD. Due to the slow native state *cis-trans*-isomerization rate, a limited number of isomerizations were observed. Although both based on *jump experiments*, the protocols to measure native state rates k_{N_c, N_t} and k_{N_t, N_c} are different. The observed apparent catalytic efficiency of native state back and forth isomerization were equal. As a result, SlyD was found to act as an effective PPIase on FLNa20. Notably, all proline isomerization rates in FLNa20 are catalyzed by SlyD.

In the two steps enzymatic proline isomerization model, the unfolded substrate peptide chain binds with high affinity, stably to SlyD's chaperone domain. In this complex back and forth proline isomerization occurs at the catalytic rate k_{cat} . In the RNase T1 a *trans-cis*-isomerization step is limiting the folding rate causing a long folding time $\tau = 530 \text{ s}$. SlyD catalyzes *cis-trans*-isomerization, but binding of its chaperone domain might inhibit folding of the substrate. Michaelis-Menten type analysis of SlyD catalyzed RNase T1 folding gives an apparent catalytic efficiency of $0.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [ŽGS13].

Jump experiments in the presence of SlyD that are presented in section 10.3 were conducted in the linear regime of enzymatic proline isomerization. A fit of a linear model to the SlyD-dependent *cis-trans*-isomerization rates gives $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The results for RNase T1 and *cis* FLNa20 are fairly similar, i.e. SlyD is a highly efficient enzyme on both substrates.

The side chains of the amino acid residues that form FKBP's binding pocket are non-essential for the PPlase activity [II07]. A designed cavity of a size similar to the hydrophobic binding pocket of FKBP on barnase—a non-PPlase bacterial nuclease—creates a significant artificial PPlase activity [IKI08]. During the course of the study presented in this work, a study on SlyD from *Thermus thermophilus* revealed a number of aspects of the catalytic mechanism on proline isomerization [QWUB⁺16]. During catalysis, the substrate was anchored to SlyD towards the N-terminus of the proline. Enzymatic proline isomerization is than a C-terminal rotation by 180° .

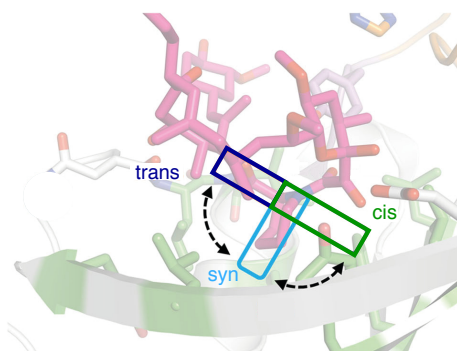


Figure 72 Cartoon representation of FK506 (as pink sticks) bound to SlyD's FKBP-domain (in white, the residues forming the hydrophobic binding pocket are green). The colored boxes indicate the position of the proline side chain in its *trans* (blue), *cis* (green) and *syn* (cyan) state. The side chain is shown in the *syn* conformation. In the *syn* state, the side chain reaches considerably deeper into the binding pocket, thus stabilizing the bound transition state. Adapted from [QWUB⁺16].

As shown in figure 72, the proline side chain reaches considerably deeper into SlyD's binding pocket at a rotational state of 90° , thus stabilizing the twisted transition states N_{syn} or U_{syn} .

P2225 undergoing isomerization in FLNa20 is located in an unstructured loop. Enzymatic native state proline isomerization can be described by a kinetic model where the loop in FLNa20 shows only weak or no interaction in the *cis* and *trans* configuration with SlyD. A stabilization of the partially rotated proline transition state in the hydrophobic cavity then mediates the catalysis of proline isomerization [QWUB⁺16].

Moreover, catalysis of native state proline isomerization is essential, if phosphorylation independent proline isomerization acts as an intrinsic conformational switch, e.g. triggering ion channel opening and closing [LBL⁺05], controlling protein autoinhibition [SRS⁺07] or modulating the mechanosensing region in FLNa20 [RMOR14]. Enzymatic proline isomerization in a folded

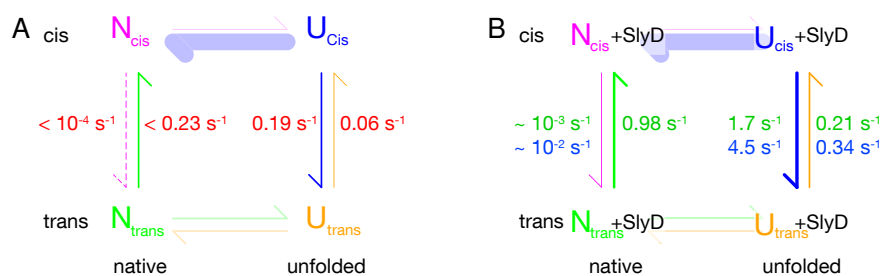


Figure 73 Kinetic network of FLNa20 with proline isomerization of the p2225. There are four stable conformations: folded and unfolded with p2225 either in cis- or trans-state. (A) shows the possible transitions and gives the measured proline isomerization rates in absence of SlyD. (B) shows the kinetic FLNa20 network in the presence of SlyD. There are no protein complexes of SlyD bound to FLNa20. The proline isomerization of p2225 in folded and unfolded FLNa20 is catalyzed by SlyD. The enzymatic proline isomerization rates are given for 1 μM (in green) and 2 μM (in blue) SlyD.

protein, experimentally supports the model of SlyD mediated proline isomerization catalysis via transiently stabilizing the transition states N_{syn} and U_{syn} .

10.8. Summary

In this chapter, *jump experiments* unveiled SlyD-dependent enzymatic proline isomerization of p2225 in FLNa20's unfolded and notably, in the native state as well. In combination with the SlyD independent un-/folding kinetics, binding of unfolded FLNa20 to SlyD appeared not to be mandatory for enzymatic proline isomerization. The PPlase mechanism does not depend on a high binding energy that stabilizes the Michaelis complex. In contrast, SlyD catalyzes proline isomerization via a transient interaction. The enzymatic proline isomerization mechanism is a preferential stabilization of the twisted transition state. Established SMFS protocols fail to study the complex multistate kinetic network of FLNa20 in presence of PPlase in all details. Kinetics of rare transitions or between distinct states with identical contour length was measured using *jump experiments*. Hence, the *jump experiment* protocol powerfully extends the SMFS toolbox.

11. Outlook: Three Force Level Jump Experiments to Assign States Kinetically

Not for all scenarios folding analysis using refolding probabilities determined during a two force level *jump experiment* is suitable. During *jump experiments*, it remains hidden whether a long-lived unfolded state, e.g. FLNa20 U_t , can refold into a weak state or not. Lifetimes of states with low folding free energy difference are too short to be well resolved in a two force level *jump experiment*. A three force level *jump experiment* protocol was used to measure U_t folding rates. During *jump experiments* with three force levels, the more stable N_c (pink in figure 74) and less stable N_t (green in figure 74) native states were distinguishable.

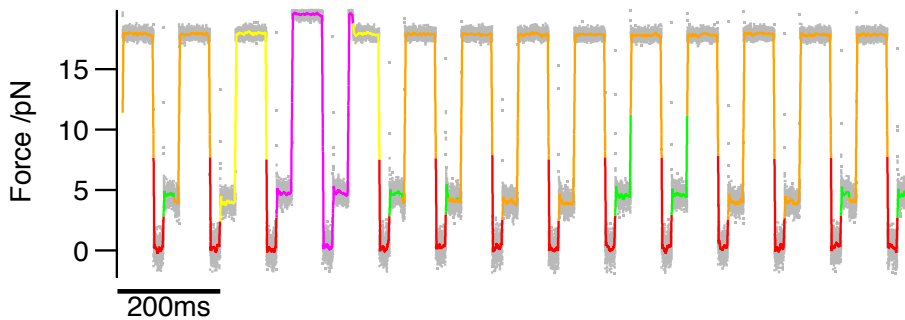


Figure 74 Un-/folding force trajectory obtained during a three force level *jump experiment*. At the mid force level of ≈ 5 pN, the N_t (green) state is resolved. Conformational transitions under low force are not resolved (red). The folded contour length under high force corresponds to N_c (purple). Isomerization is not directly observed, thus unfolded peptide following N_c is in an unknown isomerization state (in yellow). U_t is colored in orange. *Jump experiment* parameters: $\tau_h = 60$ ms, $\tau_{mid} = 30$ ms, $\tau_l = 20$ ms, $F_h = 19.5$ pN, $F_{mid} = 4.8$ ms, and $F_l = 0.3$ pN

The high force level is used to probe for the stable native state N_c , as it shows an appreciable lifetime under high forces (figure 75B). The high force also ensures the unfolding of the weaker state. Waiting under low force, the unfolded protein attempts to fold. At the mid force level, successful folding is detected. Data obtained in a three force level *jump experiment* is prepared prior to analysis as described in section 7.7.1.

In the un-/folding force trajectory created from the high force level, the state of interest, the long-lived unfolded state (orange) was assigned. Next, the corresponding assignment was transferred to the mid force un-/folding force trajectory. Folding kinetics were analyzed in these (orange) phases. Figure 75C shows a part of the mid force trajectory that corresponds to the long-lived unfolded phase (orange in figure 48A). A jump dwell time at the

mid force level, that begins in the folded state is counted as a successful folding attempt (labeled "1" in figure 75C). The weak state refolding rate under the low force was determined using equation 7.25.

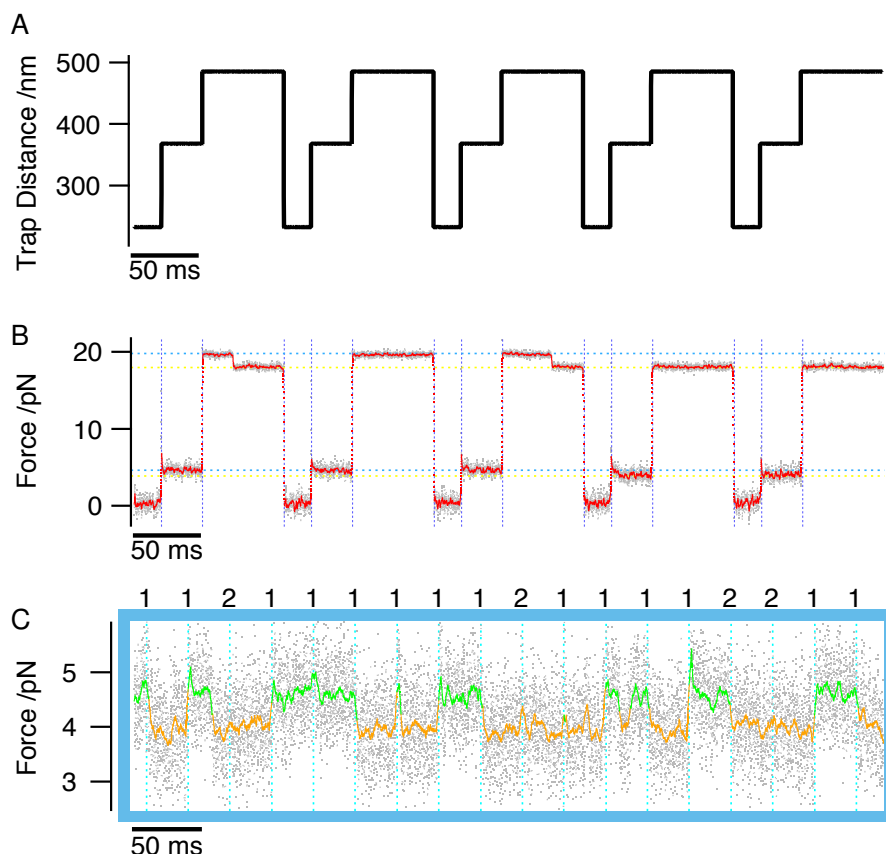


Figure 75 The three-level AOD pattern to measure U_t folding (A) and the corresponding un-/folding force trajectory (B). Full bandwidth data is shown in gray, smoothed in red. Vertical dotted cyan lines indicate the position of a force jump. Horizontal, dotted lines indicate force levels of the folded (in bright blue) and unfolded state (in yellow). Under low force, the contour length change due to folding is not resolved. After data preparation (section 7.7.1), the un-/folding force trajectory at the high force level was used to assign proline isomerization phases. (C) shows an un-/folding force trajectory section of trans-FLNa20 created from the mid force level. The colors of the smoothed trace are assigned via HMM classifier. Green: folded trans, Orange: unfolded trans. Events of refolding within the waiting time at low force are observed here and labeled with 1, unsuccessful attempts with 2.

Trans folding kinetics are not accessible in a two force level *jump experiment*. For the cis-trans assignment, conditions are used in that the native state of trans-FLNa20 is masked. U_t folding kinetics were measured using three force level *jump experiments*. To estimate the folding kinetics of trans-FLNa20 the AOD was used to regularly jump between three force levels as explained in section 7.6. Figure 76 shows data obtained during a *jump experiment* that was used to measure U_t folding under 0.39 pN in presence of 1 μ M SlyD. By jumping to 18–20 pN trans-FLNa20 was forced to unfold (figure 76A). Under forces ≈ 4.2 pN, the N_t state is observable (green in figure 76B).

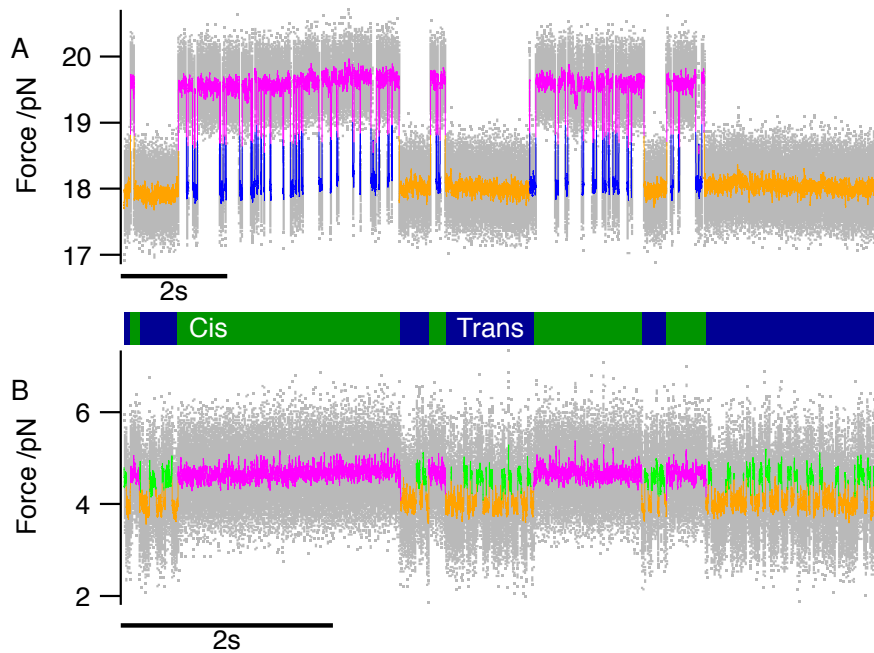


Figure 76 Typical force level time traces after data preparation (section 7.7.1) obtained during a three force level *jump experiment* (as described in section 7.5). Cis and trans phases in the high force un-/folding trajectory (A) are assigned as described in section 7.6. Gray: Full bandwidth data. The color of the smoothed data was assigned via HMM. Pink: Cis folded, Blue: Cis unfolded, Green: Trans folded, Orange: Trans unfolded. Cis and trans phases (green and blue box) are assigned to the mid force level time trace (B) to observe the refolding kinetics in trans.

The cis- and trans-phases were assigned (as explained in section 7.6) via HMM on the high force un-/folding force trajectory. The force range for the mid-level is very narrow in case of the FLNa20 construct. Under forces higher than 6 pN, a non-negligible fraction of N_t events is too short-lived to be detected. Under lower forces, the signal to noise ratio of the deflection from folding of FLNa20 declines. Figure 76B shows, that the folded and unfolded FLNa20 states are well resolved under 4–5 pN. The jump dwell time at low force was used to successfully fold FLNa20 in the majority of attempts. The 20 ms used here resulted in 75 % of the refolding attempts being successful. The estimated refolding rate of trans-FLNa20 at 0.39 pN was $(63 \pm 6) s^{-1}$. The U_t folding rate was extrapolated to $k_{U,N}^0 = (89 \pm 2) s^{-1}$ (via a fit of the Bell model to the data, section 5.3) from data obtained during passive mode SMFS experiments [RMOR14].

In summary, protein states that show identical extension in SMFS but different lifetimes are inherently challenging to assign correctly. The three force level *jump experiment* with FLNa20 show how *jump experiments* can be adapted to assign individual states and measure lifetimes and transition rates. Initially developed to speed up un-/folding kinetics of FLNa20 to observe fast enzymatic proline isomerization, *jump experiments* proved to be a powerful and versatile tool in SMFS.

Part IV

Appendix

A. Material and Methods

A.1. Supplementary Information on the Optical Tweezers Setup

The distance between the trapping centers can be chosen, such that the trapping potential does change little with changes in the bead deflection. This mode is called passive force clamp [GWAB05]

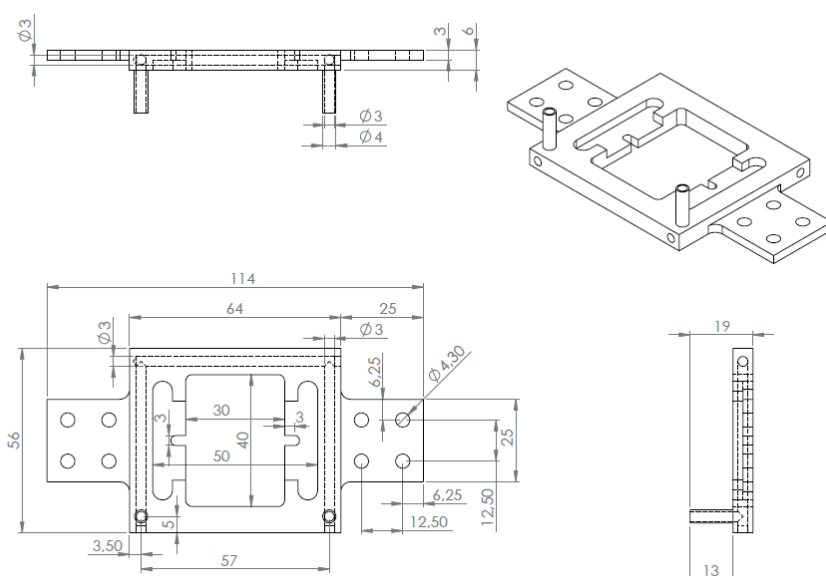


Figure 77 Sample holder cooled

A.2. Molecular cloning

A shortened mutant of FLNa20 (residue numbers are used as in the crystal structure pdbID: 2J3S) lacking the first two β -strands starting with residue 2172 was used during all the SMFS experiments throughout this work. No intermediate states are known for this construct, it shows simple two-state un-/folding kinetics for the p2225 being cis or trans with different rates. The pulling construct was inserted between two ubiquitin domains with terminal cysteines using molecular cloning techniques. The ubiquitins serve as spacer-molecules to prevent direct reaction of the terminal cysteines later used for DNA-handle coupling. The internal cysteine (Cys2199) was mutated to serine. Because ubiquitin domains are mechanically much more

FLNa20:

SlyD:

A.3. Dibenzylcyclooctyne-Maleimide (DBCO-Maleimide) Chemistry Conjugation Protocol

Oligo:

GGCAGGGCTGACGTTCAACCAGACCAGCGAGTCG-Azide

The oligo attached protein is purified by high-performance liquid chromatography (HPLC) using a YMC Dio 300 column.

A.4. Handles

As handles, 180 nm long double-stranded DNA molecules were produced by Polymerase Chain Reaction (PCR) on a lambda phage template. The sense primers were triply functionalized with biotin or digoxigenin, while the anti-sense primers contained an abasic site that caused the polymerase to fall off and leave a single-stranded overhang. This overhang was hybridized to the protein-coupled DNA oligos, which resulted in the complete DNA-protein-DNA construct.

A.5. Pooling Life Times from different Jump Experiments

For a simulated *jump experiment* time trace with 15000 observed non-enzymatic *cis* lifetimes, equation 7.26 is used to shift the log-likelihood function to represent k_{N_c, N_t} . The k_{N_c, N_t} log-likelihood maximum is within 10 % from the simulation input $5.5 \times 10^{-5} \text{ s}^{-1}$ (figure 78).

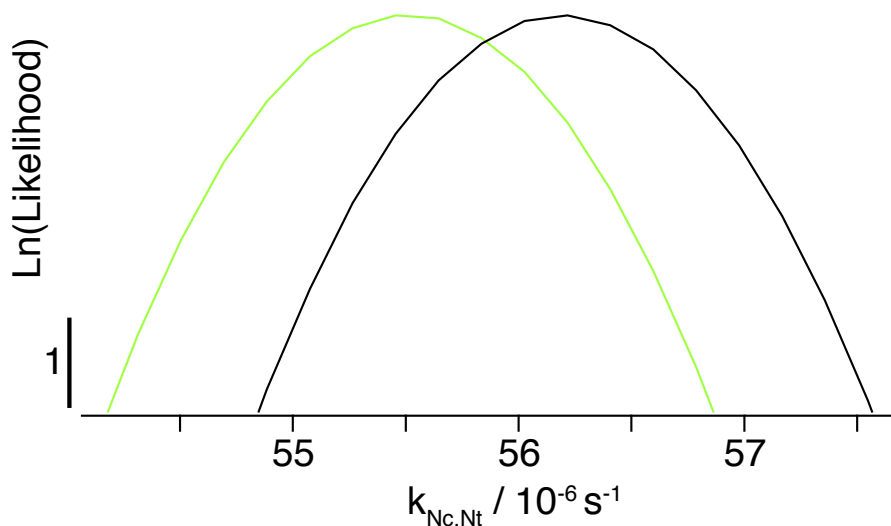


Figure 78 Native state FLNa20 *cis-trans*-isomerization rate log-likelihood for 15000 simulated, non-enzymatic FLNa20 *cis* lifetimes. Simulation input for native state *cis-trans*-isomerization rate is $55 \times 10^{-6} \text{ s}^{-1}$ [RMOR14]. Simulated protein lifetimes in green, lifetimes evaluated from the time traces from high force time traces after post-processing (see: section 7.7.1) in black.

Lifetimes from different experiments can be pooled to estimate the slow native *cis-trans*-isomerization k_{N_c, N_t} . An apparent lifetime $\tau_{N_c, t}^{\text{app}}$ is calculated as:

$$\tau_{N_{c,t}}^{\text{app}} = \frac{\tau - p_{U_c}^h \cdot k_{U_c, U_t}(F_h) - p_{U_c}^l \cdot k_{U_c, U_t}(F_l)}{p_{N_c}} \quad (\text{A.1})$$

The *MLE* native state *cis-trans*-isomerization rate k_{N_c, N_t} yields:

$$k_{N_c, N_t} = \frac{1}{\sum \tau_{N_{c,t}}^{\text{app}}} \quad (\text{A.2})$$

The convergence of the *MLE* is slower for pooled data than for data obtained during a single experiment. Thus a sufficient amount of data is challenging to obtain.

A.6. Folding Rates Determined using Overlapping Life Time Distributions

Figure 79 shows data of the high force level obtained during a *jump experiment* after preparation (section 7.7.1). The unfolded U_t and U_c lifetimes are not separated. Before determining the U_c folding rate according to section 7.5 the U_t lifetimes are subtracted. To estimate the U_t lifetime distribution, a single exponential distribution function with a cutoff is fitted to the long unfolded lifetimes. The U_c folding rate is calculated using equation 7.25 on the U_c lifetimes.

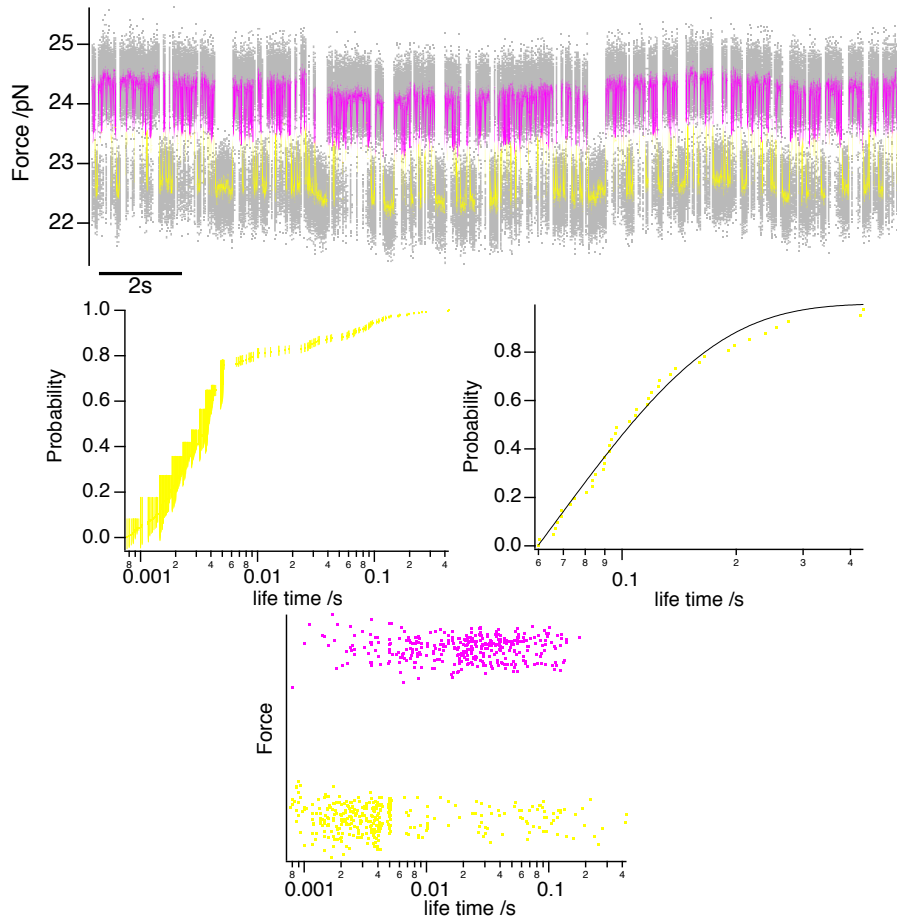


Figure 79 The top panel shows a FLNa20 un-/folding force trajectory created from a *jump experiment* high force level in presence of 4 μM SlyD. States with two different contour lengths are visible. The native folded state, due to the force assigned as N_c . The middle left panel shows unfolded lifetime probabilities that are non-separated and consist of U_t and U_c . The long lifetimes are U_t only and therefore are single exponentially distributed (middle right panel). An exponential function with cutoff is fitted (shown as a black line). The calculated U_t lifetime is subtracted from all unfolded lifetimes (as yellow dots in the bottom panel), to calculate the k_{U_t, N_t}^0 (as explained in equation 7.25).

B. Non-Equilibrium Analysis

By extrapolating the unfolding rate $k(F)$ to zero force (see section 5.2) the height of the unfolding energy barrier ΔG can be calculated as:

$$\ln k_{unf}(F = 0) = \ln \nu_0 - \frac{\Delta G}{k_B T} \quad (\text{B.1})$$

where ν_0 is an attempt frequency. For proteins that fold against a biasing force, the energy barrier height for folding can be calculated accordingly and thus the folding free energy difference can be determined.

The non-equilibrium work theorem, i.e. Jarzynski's equality [Jar97], relates non-equilibrium measurements to equilibrium properties. In non-equilibrium SMFS experiments, the fluctuations of non-equilibrium work in different repetitions W_λ ¹ is related to the difference in equilibrium free energy by:

$$\left\langle \exp \left(-\frac{W_\lambda}{k_B T} \right) \right\rangle_N = \int dW_\lambda \rho(W_\lambda) \exp \left(-\frac{W_\lambda}{k_B T} \right) = \exp \left(-\frac{\Delta G}{k_B T} \right) \quad (\text{B.2})$$

where $\langle \dots \rangle_N$ is an average over N pulling cycles and $\rho(W_\lambda)$ is the non-equilibrium work distribution [HS03]. $\rho(W_\lambda)$ depends on the schedule for varying the work parameter λ , in the constant velocity experiments the loading rate (force increase velocity). Constant velocity SMFS data has been used to determine protein folding free energy [HSK07]. The schedule for varying λ must be pre-determined to use equation B.2. Data binning can be used to overcome that the force trajectory is different in every constant velocity curve [HS01].

If un-/folding of the sample protein can be observed in a *stretch-and-relax cycle* Crooks' fluctuation theorem (CFT) is applicable [Cro99]. The non-equilibrium work distributions of the stretching $\rho(W_+)$ and relaxing $\rho(W_-)$ are used to estimate the folding-free-energy ΔG .

$$\frac{\rho(W_+)}{\rho(W_-)} = \exp \left(\frac{W - \Delta G}{k_B T} \right) \quad (\text{B.3})$$

where W corresponds to the work at which W_+ and W_- intersect, $W = \Delta G_0$ for $\rho(W_+) = \rho(W_-)$. For fast pulling scenarios, the distributions W_+ and W_- might be separated and CFT fails.

¹ The work during a pull corresponds to the area under the force–extension curve.

C. Supplemental Results

C.1. Statistics of Un-/folding Lifetimes

Thus, the folding rate distributions were analyzed in more detail.

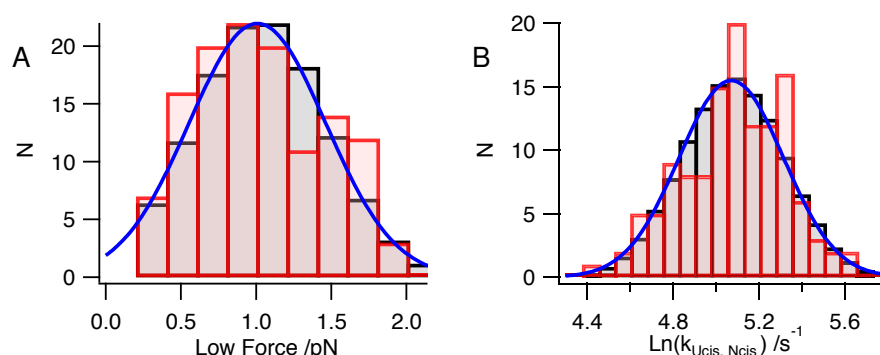


Figure 80 Histogram of the measured folding rates of cis-FLNa20 (red bars), a normal distribution with identical mean and standard deviation (black bars) and the corresponding probability density function (blue line) (A). The folding rate is expected to be exponential distributed, for more an intuitive visualization a histogram of the logarithm of the folding rates (red bars) is shown together with the histogram of a normal distribution with the same mean and standard deviation (black bars) and the probability density function (blue line) (B). The measured histograms are used in χ^2 -tests against the random histograms if they are samples of the same distribution. Results of the χ^2 -tests are shown in table 3.

Figure 80 shows histograms of the measured force and the logarithm of the calculated U_c folding rate $\ln(k_{U,N})$ (shown as red bars). Both diagrams show visual similarity with a normal distribution (shown as a blue line). If the force is normally distributed, deviations in the measured force can be considered experimental noise. A χ^2 -test¹ against a normally distributed random sample (shown as black bars) was performed on the data.

For a confidence level of 95 %, the null hypothesis is accepted (see table 3). Since the refolding rates of all experiments follow a Gaussian distribution, t-tests² on the data sets for the different SlyD concentrations were t-tested against the non-enzymatic refolding kinetics, to check whether refolding kinetics are SlyD-dependent. Results of the t-tests are shown in table 4.

¹ [Pea00]

² The t-test is used to determine if two data sets are from different normal distributions [Stu08].

Table 3 Results for the χ^2 -test of the force and the logarithm of the refolding rate distributions against Gaussian distributions with identical mean and standard deviation. If χ is smaller compared to the critical value the tested distributions are accepted equal H_0 . The confidence level is 95 %.

	Force	Fate
Critical	15.5073	24.9958
P	0.832329	0.987027
H_0	Accepted	Accepted

Table 4 Results of the t-tests for refolding rate distributions in the presence of different SlyD concentrations of SlyD. The confidence level is 95 %. All samples are from the same distribution.

$c(\text{SlyD}) / \mu\text{M}$	1	2	4
P	0.442697	0.090564	0.071736
Critical	± 2.03004	± 2.0197	± 2.08235
H_0	Accept	Accept	Accept

C.2. Cis-Trans Transition State Distance

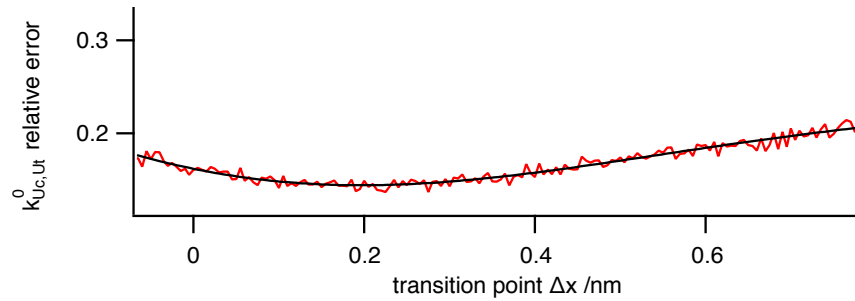


Figure 81 Unfolded FLNa20 *cis-trans* isomerization rate scattering over assumed transition point distance.

Due to previous findings, the native state *cis-trans* isomerization is assumed to be negligible $k_{N_c, N_t} \ll k_{U_c, U_t}^0$ [RMOR14]. With the observed apparent isomerization rate $k_{c,t}^{\text{app}}$ and the population probabilities at the high and low force level $p_{U_c}^h$ and $p_{U_c}^l$ the *cis-trans* isomerization rate k_{U_c, U_t}^0 is approximated:

$$k_{U_c, U_t}^0 = \frac{k_{c,t}^{\text{app}}}{p_{U_c}^h \cdot k_{U_c, U_t}(F_h) + p_{U_c}^l \cdot k_{U_c, U_t}(F_l)} \quad (\text{C.1})$$

The force-dependent *cis-trans* isomerization is described by the Bell model. A wrong assumption of transition state distance results in an increased rate error. The *cis-trans* isomerization rate error in the absence of SlyD shows a minimum (figure 81). A polynomial fit gives a minimum at 0.2 nm.

Accounting for the rather crude approach and the limited amount of data this result supports the findings in Ref. [RMOR14] and unless mentioned otherwise $\Delta x = 0.17$ nm is used.

Acronyms

AOD	acousto optical deflector
Bell model	<i>Zhurkov–Bell model</i>
<i>B-S model</i>	<i>Berkemeier-Schlierf model</i>
DBCO	dibenzocyclooctin
DNA	deoxyribonucleic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
eWLC	extensible-worm-like-chain
FLNa20	domain 20 of filamin A
HMM	Hidden Markov Model algorithm
HPLC	high-performance liquid chromatography
LASER	light amplification by stimulated emission of radiation
<i>MLE</i>	maximum likelihood estimator
NA	numerical aperture
N _c	native state of FLNa20 with p2225 being cis
NHS	N-hydroxysuccinimide
NMR	<i>nuclear magnetic resonance spectroscopy</i>
N _t	native state of FLNa20 with p2225 being trans
p2225	proline at position 2225 in filamin A
PCR	Polymerase Chain Reaction
PPIase	peptidyl-prolyl cis-trans isomerase
proline isomerization	peptidyl-prolyl cis/trans isomerization

RNA	ribonucleic acid
SlyD	sensitive – to – lysis protein D
SMFS	single-molecule force spectroscopy
SPAAC	Strain-Promoted Azide-Alkyne (copper free) Click Chemistry
TCEP	Tris(2-carboxyethyl)phosphine
U _c	unfolded peptide of FLNa20 with p2225 being cis
U _t	unfolded peptide of FLNa20 with p2225 being trans
WLC	worm-like-chain

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