How Do Chemical Denaturants Affect the Mechanical Folding and Unfolding of Proteins?

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We present the first single-molecule atomic force microscopy study on the effect of chemical denaturants on the mechanical folding/unfolding kinetics of a small protein GB1 (the B1 immunoglobulin-binding domain of protein G from Streptococcus). Upon increasing the concentration of the chemical denaturant guanidinium chloride (GdmCl), we observed a systematic decrease in the mechanical stability of GB1, indicating the softening effect of the chemical denaturant on the mechanical stability of proteins. This mechanical softening effect originates from the reduced free-energy barrier between the folded state and the unfolding transition state, which decreases linearly as a function of the denaturant concentration. Chemical denaturants, however, do not alter the mechanical unfolding pathway or shift the position of the transition state for mechanical unfolding. We also found that the folding rate constant of GB1 is slowed down by GdmCl in mechanical folding experiments. By combining the mechanical folding/unfolding kinetics of GB1 in GdmCl solution, we developed the “mechanical chevron plot” as a general tool to understand how chemical denaturants influence the mechanical folding/unfolding kinetics and free-energy diagram in a quantitative fashion. This study demonstrates great potential in combining chemical denaturation with single-molecule atomic force microscopy techniques to reveal invaluable information on the energy landscape underlying protein folding/unfolding reactions.

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Introduction

Understanding the mechanism of protein folding and unfolding remains one of the most challenging questions in life sciences.1–3 It requires not only detailed knowledge of different species, transient or long-lived, involved in folding/unfolding reactions but also detailed kinetic information that connects these species.4 During the folding/unfolding reactions, a large number of noncovalent interactions form or break on various time scales, leading to distinct processes/phases in protein (un)folding reactions, such as hydrophobic collapse and secondary structure formation. A wide range of experimental techniques have been developed to probe the folding/unfolding processes, each relying on a specific spectroscopic probe,1,2 such as circular dichroism and tryptophan fluorescence, and a specific perturbation method to perturb and initiate protein (un)folding processes, with thermal and chemical denaturation methods being the two most frequently used methods. However, due to the magnitude of the complexity involved in the folding and unfolding of proteins, different probes and different perturbation methods may probe different aspects of protein folding/unfolding processes that are not necessarily in line with each other and can sometimes lead to very different conclusions. This scenario represents an interesting analogy with an ancient Chinese fable of blind men’s view of an elephant.5 Therefore, it is crucial to use multiple probes to investigate the folding/unfolding processes in order to achieve a more complete and nonbiased picture of the protein folding/unfolding...
processes. Moreover, the fact that protein folding/unfolding processes are not synchronized calls for single-molecule approaches to this classical problem. The recently developed arena of single-molecule techniques has provided unprecedented opportunities for looking into protein folding/unfolding processes at the single-molecule level. For example, single-molecule fluorescence spectroscopy on a small cold shock protein has enabled the calculation of limits on the polypeptide reconfiguration time, which leads to the determination of limits on the height of the free-energy barrier to folding.

Among the single-molecule techniques, single-molecule atomic force microscopy (AFM) is of particular interest and has opened up new avenues toward experimental investigations of protein (un)folding dynamics. In single-molecule AFM experiments, stretching force is used as a perturbation to trigger mechanical unfolding reactions. Proteins are forced to undergo unfolding reactions along a predefined reaction coordinate determined by the stretching force acting on the protein of interest. The mechanical stability of a protein provides valuable information about the energy landscape underlying the folding/unfolding processes. This unique experimental setting has revealed novel information on protein (un)folding dynamics, which is otherwise not possible to observe. For example, single-molecule AFM experiments have revealed complex continuous folding trajectories for ubiquitin under a small but constant stretching force. Stretching proteins from different directions provides a realistic possibility of mapping the folding/unfolding energy landscape at unrivalled details and dimensions.

The mechanical stability of a protein is not only determined by its own three-dimensional structure but also sensitive to environmental perturbation. Both theoretical and experimental studies have revealed that temperature and pH changes will cause a dramatic alteration of a protein’s mechanical stability. Hence, single-molecule AFM provides opportunities for combining with other perturbation methods to investigate how the mechanical folding and unfolding pathways are modulated by additional perturbation, which will provide valuable information about the structure of the mechanical unfolding/folding transition state. Recently, single-molecule AFM has been used in combination with thermal softening to investigate how thermal perturbation affects the mechanical unfolding pathway of proteins. Chemical denaturation is a method that is also widely used to study protein folding/unfolding kinetics. Surprisingly, the mechanism by which chemical denaturants weaken the mechanical stability of proteins and affect the mechanical (un)folding pathway along the direction defined by the stretching force remains unexplored. Here we use a small protein, the B1 immunoglobulin-binding domain of protein G from *Streptococcus* (referred to as GB1 hereafter), as a model system to present the first quantitative study of the effects of the chemical denaturant guanidinium chloride (GdmCl) on the mechanical (un)folding pathways and kinetics. We found that the mechanical stability of GB1 is weakened by denaturants. The mechanical unfolding rate is sped up by the presence of denaturants, while the position of the mechanical unfolding transition state remains unaltered. The mechanical folding of GB1 is also slowed down by GdmCl. Based on our mechanical folding/unfolding results, we developed the “mechanical chevron plot,” which is analogous to chevron plot in chemical denaturation studies, to quantify the effect of GdmCl on the folding/unfolding kinetics and energetics.

### Results

**GB1 is mechanically weakened by chemical denaturants**

GB1 is a classical paradigm for protein folding/unfolding studies. It is a small α/β protein with only 56 amino acid residues composed of a four-stranded β sheet packed against a long α helix (Fig. 1a). The folding/unfolding kinetics of GB1 has been well characterized by both thermal and chemical denaturation methods. In order to study the mechanical stability of GB1, we constructed a polypeptide of (GB1)_8 that is composed of eight identical tandem repeats of GB1. As we have reported previously, stretching polypeptide (GB1)_8 resulted in force-extension curves of the characteristic sawtooth pattern appearance (red trace in Fig. 1b), in which individual force peaks correspond to the sequential mechanical unfolding event of each individual GB1 domain in the polyprotein chain. The last force peak corresponds to the stretching and subsequent detachment of the completely unfolded polypeptide chain from either cantilever tip or glass substrate. In phosphate-buffered saline (PBS), GB1 unfolds at an average force of 178 pN at a pulling speed of 400 nm/s. To study how a chemical denaturant alters the mechanical stability of GB1 quantitatively, we carried out mechanical unfolding experiments of GB1 in the presence of different concentrations of the denaturant GdmCl. It is known that a high denaturant concentration will increase the viscosity of the solution and hence increase the hydrodynamic drag force experienced by the AFM cantilever in AFM measurements. To minimize the adverse effect of high viscosity on AFM experiments, we chose the more powerful denaturant GdmCl instead of urea, since GdmCl can unfold proteins at lower concentrations as compared with urea. As shown in Fig. 1b, the mechanical unfolding of (GB1)_8 in the presence of GdmCl results in sawtooth-like force-extension curves. A worm-like chain model of polymer elasticity fitted to consecutive unfolding events revealed a contour length increment identical to that in the absence of the denaturant, indicating that chemical denaturants do not affect the contour length increment of GB1. This result suggests that
chemical denaturants do not change the nature of the two-state unfolding of GB1, and that the unfolding events observed in the sawtooth pattern correspond to the complete unfolding of GB1. Despite the identical contour length increments, the mechanical unfolding of GB1 domains occurred at lower forces with increasing GdmCl concentrations (Fig. 1b). The unfolding force histograms of GB1 under seven different GdmCl concentrations (0.6 M, 0.8 M, 1 M, 1.25 M, 1.5 M, 2 M and 2.25 M), as well as in PBS, are shown in Fig. 2a. All the unfolding force histograms showed unimodal distributions with similar widths of ~55 pN. We observed a systematic shift of average unfolding force toward a lower value as the GdmCl concentration increased: the average unfolding forces of GB1 decrease from 180 pN in PBS to 97 pN at a GdmCl concentration of 2.25 M (Fig. 2b). Note that the decreasing unfolding forces of GB1 are almost linear with respect to the concentration of GdmCl. Since the unfolding force is a direct measure of a protein’s mechanical stability, these results indicate that chemical denaturants weaken the mechanical stability of GB1 domains in a concentration-dependent fashion.

The mechanical unfolding distance is unaltered by denaturants

The mechanical unfolding of proteins is determined by the underlying energy landscape. The mechanical unfolding energy landscape is characterized by two important parameters: the energy barrier for mechanical unfolding, and the distance between the folded state and the transition state ($\Delta x_u$). The mechanical unfolding rate constant at a given force $F$ follows the following relationship:30,31

$$\alpha(F) = \nu \cdot e^{-\frac{\Delta G_u}{k_B T}} = \alpha_0 \cdot e^{-\frac{F - \Delta x_u}{k_B T}}$$

where $\nu$ is the prefactor, $\Delta G_u$ is the unfolding free-energy barrier, $\alpha_0$ is the spontaneous unfolding rate constant, and $k_B$ is the Boltzmann constant.

Fig. 2. The relationship of the mechanical stability of GB1 with the denaturant concentration. (a) The unfolding force histograms of GB1 protein at different denaturant concentrations. The GdmCl concentration is indicated above each histogram. The unfolding forces of GB1 span a range of approximately 150 pN at all GdmCl concentrations, while the average unfolding force of GB1 decreases with increasing GdmCl concentration. Solid lines refer to the Gaussian fit to experimental data. The number of events in each histogram, from bottom to top, is 3190, 1785, 767, 1428, 1304, 1032, 1133 and 136, respectively. (b) The average unfolding force of GB1 decreases with increasing GdmCl concentration. The solid line is a linear fit to the experimental data.
constant along the reaction coordinate defined by the stretching force, $k_B$ is the Boltzmann constant and $T$ is temperature. To quantitatively describe the effect of chemical denaturants on mechanical unfolding, we determine $\Delta x_u$ and $\alpha_0$ in the presence of chemical denaturants.

Previous single-molecule AFM experiments on proteins have demonstrated that the analysis of unfolding force distributions provides information about the underlying free-energy landscape. The width of the force histogram is directly related to the distance between the folded state and the transition state ($\Delta x_u$) along the mechanical unfolding reaction coordinate.\textsuperscript{31} As shown in Fig. 2a, the width of unfolding force histograms at different GdmCl concentrations is around 55 pN, which is very close to that for GB1 in PBS, indicating that the unfolding distance of GB1 is unaltered by chemical denaturants. To further confirm this observation, we also measured the pulling-speed dependence of unfolding forces of GB1 in the presence of GdmCl. Figure 3 shows the average unfolding forces of GB1 as a function of pulling speeds (ranging from 47 nm/s to 5000 nm/s) at a GdmCl concentration of 1.0 M. For comparison, the pulling-speed dependence of the average unfolding forces of GB1 in PBS is also shown (gray symbols). As expected, the two curves are parallel with each other, further confirming that the unfolding distance of GB1 at different GdmCl concentrations remained the same. Using a standard Monte Carlo simulation procedure and modeling the mechanical unfolding of GB1 as a two-state process, we reproduced the average unfolding forces at difference pulling speeds using an unfolding distance of 0.17 nm, the same as that of GB1 in PBS, with a spontaneous unfolding rate constant of 0.1 s\textsuperscript{-1}, which is 2.5 times faster than that in PBS.

### Chemical denaturants speed up the mechanical unfolding rate constant

Since the unfolding distance at different GdmCl concentrations remained unchanged as compared with that for GB1 in PBS, we extracted the unfolding rate constant at zero force for GB1 at different GdmCl concentrations using Monte Carlo simulation. We found that the spontaneous unfolding rate constant $\alpha_0$ of GB1 increases with increasing GdmCl concentration, from 0.039 s\textsuperscript{-1} in PBS to 0.42 s\textsuperscript{-1} in 2.25 M. It is evident that the chemical denaturant facilitates the mechanical unfolding reaction by speeding up the unfolding rate constant and has little effect on the unfolding distance. This result suggests that chemical denaturants do not alter the mechanical unfolding pathway for GB1.

### Chemical denaturants slow down the folding reaction of GB1

In ensemble denaturation studies, the folding rate constant of proteins is demonstrated to be slowed down upon the addition of denaturants. However, in single-molecule AFM folding experiments, the folding of proteins starts from an extended unfolded state other than a compacted unfolded state as in ensemble studies.\textsuperscript{12} The effect of chemical denaturants on the folding reaction of proteins being tethered may be different.\textsuperscript{32} Despite its importance, this question has not been probed before. We used a well-established double-pulse protocol\textsuperscript{10,27} to measure the mechanical folding kinetics of GB1 at zero force at different GdmCl concentrations. First, a polyprotein of GB1 was extended to count the total number of GB1 domains, $N_{\text{total}}$, in the polyprotein chain being picked up and stretched by the AFM tip. Then, the unfolded polyprotein chain was relaxed quickly to zero extension (usually within 2 ms) before it was detached from either the AFM tip or the substrate. The dead time of our mechanical folding experiment depends on how fast we can relax the molecule to zero extension, which is analogous to the mixing time in stopped-flow experiments. After the polyprotein had been relaxed at zero extension for a variable period of time, $t$, it was stretched again to count the number of domains folded ($N_{\text{refold}}$) during the waiting time, $t$. It was observed that the folding probability, $N_{\text{refold}}/N_{\text{total}}$, depends exponentially on the length of the relaxation time, $t$, at all GdmCl concentrations (Fig. 4), despite the fact that the folding was slowed down.

![Fig. 3. The mechanical unfolding distance is unaltered by the denaturant. The unfolding force of GB1 at a GdmCl concentration of 1.0 M is plotted against the pulling speed (black squares). The number of events for each data point, from left to right, is 50, 93, 81, 168, 148, 180, 136, 160, 137, 169, 133, 97 and 204, respectively. Error bars indicate the standard deviation of the unfolding forces. The unfolding kinetics of GB1 in 1.0 M GdmCl can be reproduced adequately by Monte Carlo simulations (black line) using an unfolding distance $\Delta x_u$ of 0.17 nm, the same as that for GB1 in PBS, and a spontaneous unfolding rate constant $\alpha_0$ of 0.1 s\textsuperscript{-1}. For comparison, the unfolding kinetics of GB1 in PBS, as well as its Monte Carlo fit, are also plotted (gray triangles and gray line). The two curves are parallel with each other, indicating that the mechanical unfolding distances $\Delta x_u$ are the same.](image-url)
dramatically with the increase in GdmCl concentration. As shown in Fig. 4, the folding probability of GB1 in 15 ms decreased from ∼100% at a GdmCl concentration of 0.6 M to ∼30% at a GdmCl concentration of 1.25 M. Since the unfolding rate constant \( \alpha_0 \) at zero force is significantly smaller than the folding rate constant \( \beta_0 \), we treated the folding reaction of GB1 as a first-order kinetics, \( \frac{N_{\text{refold}}}{N_{\text{total}}} = 1 - \exp(-\beta_0 t) \), where \( \beta_0 \) is the folding rate constant of GB1 at zero force. Fitting this function to our measured folding kinetics data measures the folding rate constants of GB1 at different GdmCl concentrations. The folding rate constant decreases from 720±120 s\(^{-1}\) in PBS to 3.3±0.4 s\(^{-1}\) at a GdmCl concentration of 1.25 M. It is clear that chemical denaturants significantly slowed down the folding reaction of GB1 being tethered between the AFM tip and the substrate.

Discussion

“Mechanical chevron plot” quantitatively describes the effects of chemical denaturants on mechanical unfolding and folding reactions.

In order to reveal the effect of denaturants on mechanical folding/unfolding reactions in a systematic fashion, we plot the measured mechanical folding and unfolding rate constants against the concentration of the chemical denaturant GdmCl. As shown in Fig. 5, the logarithms of both the spontaneous mechanical unfolding rate constant \( \alpha_0 \) and the mechanical folding rate constant \( \beta_0 \) at zero force depend on the denaturant concentration in a linear fashion: \( \log \beta_0 \) decreases linearly as GdmCl concentration increases following the relationship, \( \ln \beta_0(\text{denaturant}) = \ln \beta_0(\text{PBS}) - m_u[GdmCl]/RT \), while \( \ln \alpha_0 \) increases linearly as GdmCl concentration increases following a similar relationship, \( \ln \alpha_0(\text{denaturant}) = \ln \alpha_0(\text{PBS}) + m_u[GdmCl]/RT \), where \( R \) is the gas constant. Since free-energy barrier is linearly proportional to \( \ln k \) (with \( k \) being the rate constant), these results indicate that the free-energy barriers for mechanical unfolding and folding, \( \Delta G_u \) and \( \Delta G_f \), change linearly with respect to GdmCl concentration.

Fig. 4. The refolding kinetics of GB1 at different GdmCl concentrations. The folding probability of GB1 is plotted against the relaxation time, \( t \). The folding probability of GB1 increases exponentially with increasing relaxation time at all GdmCl concentrations. Open and filled symbols represent the data from two independent experiments for each GdmCl concentration. Fitting of the data to the function, \( P(t) = 1 - \exp(-\beta_0 t) \), measures the folding rate constant of GB1 at different GdmCl concentrations (solid line): 425±29 s\(^{-1}\) at 0.6 M, 36.3±6.9 s\(^{-1}\) at 0.8 M, 5.6±1.1 s\(^{-1}\) at 1.0 M and 3.3±0.4 s\(^{-1}\) at 1.25 M. The folding rate constant of GB1 decreases with increasing GdmCl concentration.

Fig. 5. The mechanical chevron plot quantitatively describes the effect of chemical denaturant on the mechanical unfolding/folding kinetics. The natural logarithms of the mechanical folding (in blue) and unfolding rate constants (in red) at different GdmCl concentrations are plotted against the concentrations of GdmCl. For comparison, the folding rate constants of GB1 measured by stopped-flow experiments are also plotted (in gray; taken from Ref. 22). The mechanical unfolding rate constant increases with increasing GdmCl concentration, while the mechanical folding rate constant decreases with increasing GdmCl concentration.
that the chemical denaturant speeds up the mechan-
ical unfolding reaction similarly to the chemical unfolding reaction. The measured spontaneous unfolding rate constants for mechanical and chemical unfolding reactions are indistinguishable from each other, and so are the $m_n$ values. These results indicate that the chemical denaturant softens the mechanical unfolding barrier of GB1 in the same scale as it does on the chemical unfolding barrier, and that the mechanical unfolding pathway of GB1 is likely to coincide with or be part of the ensemble of chemical unfolding pathways.

Chemical unfolding experiments using stopped-
flow spectrofluorimetry probe the change in trypto-
phan fluorescence upon disruption of the hydro-
phobic core of proteins, which is global in nature. In
contrast, mechanical unfolding probes a directional
unfolding pathway defined by the stretching force; thus, it is of local nature. Due to the different natures of the two unfolding pathways, generally, the two are not necessarily the same. The finding that the mechanical unfolding pathway of GB1 coincides with the chemical one represents a unique case in which mechanical and chemical unfolding experiments share common kinetic features. Similar find-
ings have been reported on wild-type Ig domains from titin.\textsuperscript{10,33–35} Protein G is a bacterial surface pro-
tein, and its biological function is still unknown.\textsuperscript{36}
Hence, it is unclear whether this striking coincidence between the mechanical and chemical unfolding rates for GB1 carries any physiological significance. We speculate that this striking coincidence for GB1 is more likely to be a structural consequence rather than a functional one. A common feature of the Ig domains of titin and GB1 is their highly native-like transition states for both mechanical and chemical unfolding pathways. It remains to be demonstrated whether such coincidences are also true for mechanical and chemical unfolding pathways for GB1-like domains, such as protein L.\textsuperscript{13} Previous single-
molecule AFM studies revealed that the coincidence only held for wild-type Ig domains\textsuperscript{10,33} and that such a coincidence between the two unfolding pathways was observed to disappear in point mutants of Ig domains.\textsuperscript{37,38} It remains to be seen whether a similar breakdown of such a coincidence will also occur for GB1.

Moreover, the mechanical softening effect we observed here indicated that the unfolding ener-
getics in mechanical unfolding experiments is also affected by the denaturing condition. As demon-
strated before, mechanical unfolding is anisotro-
pic.\textsuperscript{13,39,40} The mechanical unfolding kinetics of the same protein can show dramatically different behaviors when the protein is pulled and unfolded from different directions. Hence, the softening effect we reported here for stretching GB1 from its N- and C-termini may not be extrapolated to other stretching and unfolding directions. The effect of chemical denaturant on individual mechanical unfolding pathways will have to be examined on an individual basis.

Thermal softening of the mechanical stability of proteins was investigated by various groups.\textsuperscript{14–16} It

Mechanical unfolding kinetics is affected by chemical denaturants similarly to chemical unfolding

To compare the effects of chemical denaturants on mechanical and chemical unfolding pathways, we also plotted a chemical chevron plot on GB1 measured using stopped-flow spectrofluorimetry (taken from Ref. 22). It is surprising to find that extrapolation of the unfolding arm of the chemical chevron plot superimposes on the unfolding arm of the mechanical chevron plot. This result suggests that the chemical denaturant speeds up the mechan-
ical unfolding reaction similarly to the chemical unfolding reaction. The measured spontaneous unfolding rate constants for mechanical and chemical unfolding reactions are indistinguishable from each other, and so are the $m_n$ values. These results indicate that the chemical denaturant softens the mechanical unfolding barrier of GB1 in the same scale as it does on the chemical unfolding barrier, and that the mechanical unfolding pathway of GB1 is likely to coincide with or be part of the ensemble of chemical unfolding pathways.

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Thermal softening of the mechanical stability of proteins was investigated by various groups.\textsuperscript{14–16} It
was observed that increasing temperature reduced the mechanical stability of the proteins under investigation. Moreover, Schlierf and Rief also observed that increasing the temperature led to a gradual shift of the mechanical unfolding transition state away from the folded state for ddFLN4, resulting in an increase in unfolding distance. They suggested that the increase in the unfolding distance of ddFLN4 from a temperature of 5 °C to a temperature 37 °C reflects a shift of the mechanical resistance of the protein from hydrogen-bonding-dominated interactions to hydrophobic interactions. In contrast, such effects were not observed in our mechanical unfolding experiment of GB1 in GdmCl solution. Instead, we found that the unfolding distance remains constant at all GdmCl concentrations. We propose that the nature of the interactions that are crucial to the mechanical unfolding of GB1 remains unchanged in denaturing conditions, while their strength is weakened by chemical denaturants.

Although an alternative model (alteration of hydrophobic interaction model) has been proposed, the predominant view of denaturant-induced protein unfolding is based on the so-called direct interaction model, which suggests that denaturants unfold proteins by direct interactions with polypeptide chains by forming hydrogen bonds and hence disrupting or weakening native hydrogen bonds present in native folded structures of proteins. Simulations showed that the mechanical resistance of GB1 mainly comes from the hydrogen bonding between the two terminal force-bearing strands of GB1, the very interactions that are weakened by chemical denaturants. This qualitatively explains why the mechanical and chemical unfolding kinetics show similar responses to chemical denaturants. Since hydrogen bonding remains the dominant interaction responsible for the mechanical unfolding barrier in the presence of GdmCl, the mechanical unfolding transition state does not change.

The effect of chemical denaturants on mechanical refolding

The mechanical folding rate constant of GB1 is slowed down by GdmCl and shows a stronger logarithmic dependence on GdmCl concentration than that measured in stopped-flow experiments, revealing the difference between chemical and mechanical folding pathways. In chemical folding studies, the folding reaction is initiated from a denatured state, which is not necessarily random and may contain residual structures. In contrast, the folding reaction in mechanical folding studies is initiated from a well-defined fully stretched state. The experimental setting in single-molecule AFM folding experiments removes the complication of the existence of residual structures of the proteins in a denatured state and involves a collapsing phase into the kinetics, which corresponds to the collapse of the fully extended protein chain to the random coil state when the force is relaxed to zero. Therefore, carrying out folding experiments in the presence of chemical denaturants has the potential to directly dissect the influence of the chemical denaturants on the hydrophobic collapse process, as well as on subsequent folding processes.

Conclusion

We present the first single-molecule AFM study on the effect of chemical denaturants on the mechanical folding/unfolding kinetics of a small protein GB1. Upon increasing the GdmCl concentration, we observed a systematic decrease in the mechanical stability of GB1, indicating the softening effect of the chemical denaturant on the mechanical stability of proteins. This mechanical softening effect originates from the reduced free-energy barrier between the folded state and the unfolding transition state, which decreases linearly as a function of the denaturant concentration. Chemical denaturants, however, do not shift the mechanical unfolding pathway or alter the distance between the folded state and the transition state.

We also found that the folding rate constant of GB1 is slowed down by GdmCl in mechanical folding experiments. Combining the mechanical folding/unfolding kinetics of GB1 in GdmCl solution, we developed the “mechanical chevron plot” as a general tool to understand how chemical denaturants influence the mechanical folding/unfolding kinetics and free-energy diagram in a quantitative fashion. This study demonstrates great potential in combining chemical denaturation with single-molecule AFM techniques to reveal the features of the mechanical unfolding transition state. We anticipate that the combination of single-molecule AFM, protein engineering, chemical denaturation and molecular dynamics simulations will provide invaluable information regarding the structure of the mechanical unfolding transition state and will help to map the energy landscape underlying protein folding/unfolding reactions.

Materials and Methods

Protein engineering and expression

The (GB1)8 polyprotein, containing eight tandem repeats of GB1 domains, was engineered, expressed and purified as described elsewhere. Stopped-flow spectrofluorimetry experiments on (GB1)8 polyprotein indicated that GB1 domains behave independently of each other (data not shown), in agreement with other polyprotein studies. The experimental setting in single-molecule AFM folding experiments removes the complication of the existence of residual structures of the proteins in a denatured state and involves a collapsing phase into the kinetics, which corresponds to the collapse of the fully extended protein chain to the random coil state when the force is relaxed to zero. Therefore, carrying out folding experiments in the presence of chemical denaturants has the potential to directly dissect the influence of the chemical denaturants on the hydrophobic collapse process, as well as on subsequent folding processes.

Force spectroscopy of single proteins

All the single-molecule force measurements were performed with a custom-built atomic force microscope, as described. The cantilevers were calibrated in PBS solution using the equipartition theorem, with an average
error of 10%. In order to minimize errors from calibration, for all the experiments, around 50 curves of (GB1)$_8$ unfolding were obtained in PBS before switching the buffer to GdmCl solution. The average forces of these unfolding events served as internal calipers. The unfolding forces of GB1 in GdmCl solution were corrected based on the difference between the unfolding forces of GB1 in PBS in individual experiments and the average unfolding forces of all the experiments.

In a typical unfolding experiment, 1 µL of GB1 solution with a concentration of 741 ng/µL was dropped to a freshly cleaned glass coverslip containing ~50 µL of PBS or GdmCl solution and stabilized for ~10 min before measurement. The pulling speed was 400 nm/s for all the unfolding experiments, except when reported otherwise. For refolding experiments, only the cantilevers with a minimum drift were used. During the refolding experiments, we checked the starting position from time to time and adjusted the piezo position to balance the drift accordingly to ensure that the polyprotein was relaxed to zero extension.

Monte Carlo simulations

The mechanical unfolding of GB1 domains was modeled as an all-or-none process with force-dependent rate constants, in which only the folded and the unfolded states will populate during the reaction. The force-dependent unfolding rate constant can be described as: $\alpha(F, \text{GdmCl}) = \alpha_0(\text{GdmCl}) \exp(F \Delta x_u / k_B T)$, where $k_B$ is the Boltzmann constant, $T$ is the absolute temperature (in Kelvin), $\alpha(F, \text{GdmCl})$ is the unfolding rate constant at a stretching force of $F$ in GdmCl solution, $\alpha_0(\text{GdmCl})$ is the unfolding rate constant at zero force in GdmCl solution and $\Delta x_u$ is the distance between the folded state and the transition state along the direction of the force. Monte Carlo simulations were carried out in accordance with published procedures. The unfolding rate constants at different GdmCl concentrations were obtained by simulations with a fixed unfolding distance of 0.17 nm.

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