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**Article:**

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Unfolding dynamics of proteins under applied force

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Understanding the mechanisms of protein folding is a major challenge that is being addressed effectively by collaboration between researchers in the physical and life sciences. Recently, it has become possible to mechanically unfold proteins by pulling on their two termini using local force probes such as the atomic force microscope. Here, we present data from experiments in which synthetic protein polymers designed to mimic naturally occurring polyproteins have been mechanically unfolded. For many years protein folding dynamics have been studied using chemical denaturation, and we therefore first discuss our mechanical unfolding data in the context of such experiments and show that the two unfolding mechanisms are not the same, at least for the proteins studied here. We also report unexpected observations that indicate a history effect in the observed unfolding forces of polymeric proteins and explain this in terms of the changing number of domains remaining to unfold and the increasing compliance of the lengthening unstructured polypeptide chain produced each time a domain unfolds.

Keywords: protein folding; atomic force microscope; mechanical unfolding; force; single molecule; mechanical resistance

1. Introduction

The first experiments involving the mechanical unfolding of a protein were performed in Ikai’s laboratory using a strategy that involved chemical derivitization of the tip and substrate (Mitsui et al. 1996). Since then proteins have been mechanically unfolded using laser tweezers (Kellermayer et al. 1997; Tskhovrebova et al. 1997) and the atomic force microscope (AFM) (Rief et al. 1997; Carrion-Vazquez et al. 1999a; Best et al. 2001; Brockwell et al. 2002). The AFM comprises a cantilever of known stiffness, the deflection of which under applied force is measured with angstrom accuracy using an optical lever (see, for example, http://www.tmmicro.com/spmguide/contents.htm). Mechanical unfolding experiments typically record the applied force, calculated using the spring constant of the cantilever and the position of the cantilever tip.

One contribution of 14 to a Discussion Meeting ‘Slow dynamics in soft matter’.

Mechanical protein-unfolding experiments have been most successfully applied to polymeric proteins, i.e. proteins that comprise a linear sequence of, sometimes different, domains. The first polymeric protein to be mechanically unfolded was the giant muscle protein titin (Tsukrovberova et al. 1997; Rief et al. 1997). This protein consists of ca. 300 immunoglobulin (Ig) and fibronectin type-III domains as well as a 163–2174 residue-disordered region rich in P, E, V and K amino acids (Labeit & Kolmerer 1995), thought to be critically important to the mechanical properties of the polymer (Linke et al. 1998; Li et al. 2001, 2002). These experiments showed that individual domains could be observed to unfold abruptly at a critical ‘unfolding force’ in the range 50–300 pN, dependent on the pulling speed. The major drawback of studying natural polyproteins lies in their heterogeneity; interpretation of the unfolding data is limited by the presence of hundreds of different protein domains in the polymer. Thus, synthetic polyproteins, or concatamers, have been developed, which contain a controlled sequence of one type or a few different types of domain joined by amino-acid linkers (Carrion-Vazquez et al. 1999a; Best et al. 2001; Brockwell et al. 2002) or disulphide bridges (Yang et al. 2000). Several groups have chosen to study the 27th Ig domain of titin (I27), comprising 89 amino acids, and this is now by far the most extensively studied protein by mechanical unfolding experiments and theoretical studies (Lu et al. 1998; Fisher et al. 2000). The method of mechanical unfolding has also been applied to several other naturally occurring modular ‘beads on a string’ proteins: tenascin (Oberhauser et al. 1998), spectrin (Rief et al. 1999), fibronectin (Oberdörfer et al. 2000) and abalone shell protein (Smith et al. 1999).

This handful of studies has yielded some interesting and important results, not all of which are in agreement. The predicted pulling-speed dependence of the unbinding force of ligand:receptors (Merkel et al. 1999) has been shown to be applicable to forced protein unfolding. The measured dependence of unfolding force on pulling speed has also allowed the height of the unfolding and folding energy barriers ($\Delta G_u$ and $\Delta G_f$) and the position of the mechanical unfolding transition state (relative to the native state) to be determined. Interestingly, the intrinsic unfolding rate constants of I27 obtained by chemical denaturation and mechanical unfolding were reported to be very similar ($4.9 \times 10^{-4}$ s$^{-1}$ and $3.3 \times 10^{-4}$ s$^{-1}$, respectively), and to occur with a transition-state with a similar placement along the reaction coordinate (ca. 10% from the native state), implying that mechanical and chemical denaturation probe the same unfolding process (Carrion-Vazquez et al. 1999a).

Steered molecular dynamics simulations (Lu et al. 1998; Lu & Schulten 2000; Paci & Karplus 2000) have suggested that the occurrence of large unfolding forces in I27 results from the rupture of six hydrogen bonds between the A' and G strands, which need to be broken before the rest of the protein can be exposed to the force (see figure 1). Recent mechanical unfolding experiments using proline mutagenesis and loop insertions have supported the suggestion that the A’–G interface acts as a mechanical clamp which resists the applied force (Li et al. 2000a; Carrion-Vazquez et al. 1999b). The dependence of mechanical stability upon the presence of specific, highly localized hydrogen-bond ‘clamps’ and their geometry relative to the applied force is clearly at odds with the proposition that the chemical and mechanical unfolding pathways for this domain are identical. Indeed, chemical denaturation experiments have shown that, although the A' and G strands are disrupted in the transition state for unfolding, other regions of the protein are also significantly perturbed (Fowler & Clarke 2001).
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Figure 1. Nuclear magnetic resonance solution structure of monomeric I27. C47 and C63 are shown in a ball-and-stick representation, and the hydrogen bonds between the A' and G strands are shown as dashed lines. The figure was drawn using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt & Murphy 1994) using the coordinates from the protein data base file 1TIT (Improta et al. 1996). Individual β-strands are labelled A to G.

2. Materials and methods

In the experiments to be described here, a pentameric I27 concatamer consisting either of four copies of the double C47S,C63S mutant and a single copy of the single C63S I27 mutant as the central domain (denoted (I27)$_5^*$) or five copies of the double mutant (denoted (C47S,C63S I27)$_5$) were studied. Both of these mutations have been shown to severely destabilize the protein in chemical unfolding experiments (Brockwell et al. 2002) but they do not affect the hydrogen-bond network between the A' and G strands and would therefore not be expected to affect the observed unfolding forces.

The concatamer was constructed using a PCR-generated cassette strategy (Brockwell et al. 2002). Each I27 domain was regarded as comprising leucine 1–leucine 89 (from the original structure determination (Improta et al. 1996)). Linkers consisting of 4–6 amino-acids were inserted between domains to decrease inter-domain inter-
actions. The sequence of the linkers was designed to be as similar as possible to the natural I26-I27 and I27-I28 linkers (linker choice was constrained by restriction site sequence). Mechanical unfolding experiments were performed using a commercially available mechanical force probe (MFP-SA, Asylum Research Inc., USA). Coated unsharpened microlevers (MLCT-AUNM) were obtained from Veeco Metrology (Santa Barbara, USA). The spring-constant of each cantilever was calculated under phosphate-buffered saline (PBS) using the thermal method (Florin et al. 1995) and was typically found to be ca. 51 ± 5 pN nm$^{-1}$. Protein (0.05 mg) was reconstituted to 0.1 mg ml$^{-1}$ in sterile PBS and centrifuged (13 000 rpm, MSE, MicroCentaure). Typically, 50 µL of PBS was dropped onto a recently cleaved template stripped gold surface. 20 µL of protein solution was then added and the two solutions allowed to mix. At this protein concentration the probability of attaching a molecule to the tip is relatively low (typically 4%). However, under these conditions ca. 50% of the traces result in the attachment of a single molecule and four or more clear unfolding peaks. Mechanical unfolding experiments were performed using the AFM at pulling speeds varying from 70 nm s$^{-1}$ to 4000 nm s$^{-1}$ at a room temperature of 23.3 ± 1 ºC over a distance of 400–600 nm.

Kinetic chemical unfolding experiments were performed using an Applied Photo-physics SX.18 MV stopped-flow fluorimeter. The temperature was regulated using an external probe placed near the cuvette and maintained at 25 ºC using a Neslab RTE-300 circulating water bath. Tryptophan fluorescence was excited at 280 nm with a 10 nm bandwidth, and the emitted fluorescence was monitored at more than 320 nm. Unfolding experiments were performed by manual mix. Protein (ca. 50 µM) in native buffer (20 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$ pH 7.3, 1 mM EDTA and 2 mM DTT, or PBS, 1 mM EDTA and 2 mM DTT) was diluted 1:9 into solutions containing GnHCl. The decrease in fluorescence at 315 nm (excitation 280 nm) was monitored in a 1 cm path-length cuvette for 600 s. Kinetic transients (of the monomeric I27 protein) were fitted to a three-parameter single exponential equation using SIGMAPLOT (SPSS Inc.).

A two-state model was used to perform Monte Carlo simulations of the forced extension of the I27 constructs (Rief et al. 1998). Each domain of the molecule was initially assumed to be in the lowest energy state and therefore folded. The folding and unfolding rate constants at applied force $F$ were calculated using $a_{i,F} = a_{i,F}^0 \exp(\pm F x_i/k_BT)$, where $i = f$ or $u$ for the folding and unfolding events, respectively, and the negative sign is associated with folding. (So as to differentiate between the intrinsic rate constants for chemical and forced unfolding, the notation $a_{u,F}^0$, GnHCl or $a_{u,F}^0$, respectively, is used). The constants $x_f$ and $x_u$ represent the distance from the folded and unfolded well to the barrier, respectively (this reaction coordinate is assumed to be parallel to the stretch axis). The protein was extended with speeds from 10 to 10 000 nm s$^{-1}$ and with different values of $x_f$, $x_u$, $a_{f,F}^0$ and $a_{u,F}^0$, which are the rate constants for folding and unfolding, respectively, in the absence of applied force. The simplified worm-like chain (Bustamante et al. 1994) was used to calculate the force applied at any extension $x$ as

$$F = \frac{k_BT}{p} \left[ \frac{1}{4(1-x/L)^2} - \frac{1}{4} + \frac{x}{L} \right],$$

where $p$ is the persistence length and $L$ is the total contour length calculated as $L = zL_f + (n-z)L_u$ for $z$ folded domains of length $L_f$ and $n-z$ unfolded domains.

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each of length $L_u$, where $n$ is the total number of domains within the concatamer. At each extension the probability of folding, unfolding or extending the chain is calculated. If unfolding (folding) occurs, the chain length $L$ is increased (decreased), as described above, the cantilever extension incremented, and the probability of folding, unfolding or extending the protein re-calculated. The sequence of domain unfolding is random. As a consequence, the first domain to unfold, corresponding to the first pulling event, can be any one of those in the construct and not necessarily the first or last in the chain. The procedure is continued until all domains are unfolded. The whole calculation is then repeated 10,000 times.

Experimental force-extension data take the form of a sawtooth pattern from which the unfolding force can be measured for each unfolding event observed. For a given pulling speed, the observed unfolding forces are plotted in a force–frequency histogram and the mean unfolding force $F$ is obtained from this histogram. This process is repeated at several pulling speeds which span the dynamic range of the instrument. The unfolding force at each speed is used to construct a graph of $F$ against $\log(v)$ and this is compared with the results of the Monte Carlo simulation. The simulation parameters were varied until the experimental data were matched. Thus, the intrinsic unfolding rate constant of the monomeric species $a_{u,F}^0$ and the distance to the transition state from the native state $x_u$ can be obtained.

3. Results

(a) A comparison of mechanical and chemical unfolding

The results of chemical and mechanical unfolding experiments on I27 are shown in figure 2.

The chemical unfolding experiments were performed using both monomeric and polymeric protein to ensure that the behaviour of the protein is not affected by its placement in the polymer (Brockwell et al. 2002). Since this concatamer comprises four double-cysteine-mutant domains and a single-cysteine-mutant domain, it is not surprising that the unfolding kinetics fit well to a bi-exponential function with 80% of the amplitude corresponding to the decay noted for the double-cysteine mutant in monomeric form and 20% to that of the single-cysteine-mutant monomer. The intrinsic chemical unfolding rate constant of the concatamer (for the phase accounting for 80% of the amplitude) (i.e. the mean unfolding rate constant obtained by extrapolation to zero GnHCl concentration) is $10.6 \pm 0.7 \times 10^{-3} \text{s}^{-1}$.

It should be noted that, although the unfolding of the protein in chemical experiments does not appear to be affected by concatamerization, there is an effect on the measured intrinsic mechanical unfolding rate constant due to the polymeric state of the protein. The measured unfolding force depends on the probability of observing an unfolding event at any given extension, and this depends on the number of folded domains available to unfold (Brockwell et al. 2002). Clearly, this is a function of the number of domains in the concatamer and of how many unfolding events have already occurred at that given extension. One result of this dependence of the unfolding force on the number of folded domains remaining is that a simple extrapolation to the abscissa to obtain the intrinsic forced unfolding rate constant (i.e. the unfolding rate constant under zero applied force) is invalid since it depends on the number of domains in the original concatamer (Brockwell et al. 2002). Furthermore, a comparison of this value with that obtained by extrapolation in chemical unfolding.

Figure 2. Chemical and mechanical unfolding of I27. (a) Chemical unfolding rate profile of I27 monomers and the \((\text{I27})_5^\text{c}\) concatamer as a function of urea concentration. Open triangles, monomeric C47S,C63S I27; open circles, monomeric C63S I27. Solid lines are fits to a two-state model (Brockwell et al. 2002). Closed triangles, observed rate constant for the faster phase of \((\text{I27})_5^\text{c}\) unfolding; closed circles, observed rate constant for the slower phase of \((\text{I27})_5^\text{c}\) unfolding. Dashed lines show the best linear least squares fit for the fast and slow phases of unfolding of the concatamer. The open triangle and circle on the abscissa are the extrapolated \(k_{0u}\) for C47S,C63S I27 and C63S I27, respectively. (b) The pulling speed dependence of observed unfolding force. Mutant \((\text{I27})_5^\text{c}\) measured (open circles) unfolds at a lower force than wild-type \((\text{I27})_8\) (dashed line) (data taken from Carrion-Vazquez et al. (1999a)). All error bars are standard error of the mean. The solid line is a linear least-squares fit through \((\text{I27})_5^\text{c}\) data. Crosses show the best fit Monte Carlo simulation with parameters \(\alpha_{0,F}^u = 2.0 \times 10^{-3} \text{ s}^{-1}\) and \(x_u = 0.29 \text{ nm}\).

experiments is not a valid test of the similarity of chemical and mechanical unfolding processes since one describes a monomeric species and the other describes a concatamer. Thus, Monte Carlo simulations of the mechanical unfolding experiment, which have the monomeric intrinsic unfolding rate constant \(\alpha_{0,F}^u\) and the distance
Table 1. Comparison of the results of chemical and mechanical unfolding experiments

(The position of the transition state along the reaction coordinate in chemical unfolding experiments \(P\) was determined for the monomer in both the mutant and wild-type cases. Cysteine mutant data taken from Brockwell et al. (2002). Wild-type \((127)_{\text{WT}}^{s}\) data taken from Carrion-Vazquez et al. (1999a).)

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to the transition state from the native state \(x_{u}\) as parameters (see §2), are used to fit the data in figure 2b. This approach yields an intrinsic forced unfolding rate constant of \(2.0 \pm 0.2 \times 10^{-3} \text{ s}^{-1}\), more than five times slower than the intrinsic chemical unfolding rate constant. The results of the chemical and mechanical unfolding experiments on the mutant and wild-type I27 proteins are summarized in table 1.

The significant difference between the chemical and mechanical intrinsic unfolding rate constants that partly parametrize the unfolding energy landscapes for the two processes suggests that they occur with different unfolding mechanisms in contradiction to earlier work (Carrion-Vazquez et al. 1999a). The transition state for unfolding of the double mutant described here and the wild-type I27 (Carrion-Vazquez et al. 1999a) occurs \(ca. 10\%\) of the distance along the ‘reaction coordinate’ from the native state in both chemical and mechanical unfolding experiments. The relevance of this similarity is difficult to assess, however, due to the differing nature of the reaction coordinates. In classical chemical denaturation this coordinate is usually the accessible surface area exposed to solvent (Myers et al. 1995). The reaction coordinate in mechanical unfolding is end-to-end concatamer extension. The physical meaning of this one-dimensional quantity in terms of the actual deformation of protein structure is difficult to interpret. Comparison in structural terms of the transition state placement as measured by the two techniques is therefore not meaningful.

Whether the chemical and mechanical unfolding pathways are the same or not is an important issue, since there is a considerable body of literature reporting on chemical unfolding/refolding of proteins, and it is interesting to discuss the relatively new field of mechanical unfolding in the context of these results. It is relatively simple to test the hypothesis (that mechanical and chemical unfolding are related processes) by destabilizing/stabilizing the native state of the protein and testing whether this has a similar effect on the observed unfolding rate constants in the two experiments. For example, figure 3 shows the effect on the two unfolding pathways of the addition of sodium sulphate which is known to stabilize compact (native) states in chemical unfolding experiments.

As expected, in the chemical unfolding experiments the measured intrinsic unfolding rate constant is reduced by a factor of \(ca. 3\) by the addition of 0.4 M sodium sulphate (figure 3a). However, the data indicate that there is no measurable effect on the mechanical unfolding experiments (figure 3b). These data therefore strongly support the view that the two unfolding mechanisms differ. In addition to the difference in the effect of sodium sulphate on the two unfolding processes, the core...
mutations (C47S and C63S) have a different effect on the mechanical and chemical unfolding rate constants with respect to the wild-type protein. The effect of the mutations is to destabilize the core, which results in a sixfold decrease in the mechanical and a 22-fold decrease in the chemical unfolding rate constants with respect to those values reported for the wild-type protein (see table 1). These data show that the barrier heights for chemical and mechanical unfolding respond differently to mutation. This cannot simply be explained by changes in the thermodynamic stability of the native protein, since the native state is destabilized by 18 kJ mol$^{-1}$ upon mutation.

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(ΔΔG_{UN}), while the transition states for unfolding determined mechanically and chemically are destabilized by ca. 5 kJ mol⁻¹ and 8 kJ mol⁻¹, respectively.

Consequently, it would appear that chemical and mechanical unfolding pathways are different, at least for the I27 polymer studied here, and a similar observation was reported recently for the enzyme barnase (Best et al. 2001). A further important conclusion can be drawn from analysis of these data. The wild-type protein unfolds at a higher force at a given pulling speed than the mutant proteins (Carrion-Vazquez et al. 1999a; Brockwell et al. 2002), suggesting that the mutations have affected the mechanical sensitivity. However, as well as changing the barrier height for mechanical unfolding, the mutation also affects the parameter $x_u$ (using Monte Carlo methods $x_u^{WT} = 0.33 \pm 0.05$ nm (Best et al. 2002) and $x_u^* = 0.29 \pm 0.03$ nm (Brockwell et al. 2002)). It is the product $F x_u$ that equates to the energy required to reduce the barrier height sufficiently to allow crossing by thermal fluctuations. This product, rather than the absolute value of unfolding force, is the most accurate measure of the protein’s sensitivity to force. It can therefore be seen that at a pulling speed of 600 nm s⁻¹ the barrier for mechanical unfolding of wild-type I27 domains is reduced by 31 kJ mol⁻¹ and by 29 kJ mol⁻¹ for the mutant. Thus it is clear that these mutations have not in fact affected the mechanical stability, supporting the existing hypothesis that mechanical resistance is a locally endowed property of the protein, in the case of I27 is due to the hydrogen-bond clamp region, and not affected by mutations to the protein core.

4. The effects of unfolding history and supramolecular scaffold

It has been widely assumed that in a hetero-polyprotein the domain with the fastest $\alpha_{u,F}^0$ must unfold first under an applied load (Li et al. 2000b) and in a homopolyprotein all unfolding forces are equivalent within the limits of thermal fluctuations (Carrion-Vazquez et al. 1999a; Yang et al. 2000; Best et al. 2001; Brockwell et al. 2002). During our Monte Carlo simulations of the forced unfolding of the mutant described above it became clear that these two assumptions are not always valid. We observed first in simulation, then in experiment, that for a homopolymer (in our case constructed of copies of a double mutant C47SC63S; see §2), the lowest unfolding force was not necessarily the first unfolding event and that a more complex description involving the number of domains remaining folded and the length of unfolded polypeptide chain in the system was required.

In order to observe clearly this unexpected behaviour the unfolding forces must be reanalysed in terms of their position with the unfolding sequence, i.e. as a function of the unfolding ‘event number’ (Zinober et al. 2002). Figure 4a shows a typical unfolding trace both from experiment (data taken from experiments using (C47S, C63S I27)₅) and simulation (inset). If, instead of producing a histogram from all the unfolding events in each trace at a given pulling speed to yield a mean unfolding force, we select only the first unfolding events (denoted as #1 in the experimental data and simulation in figure 4a), then we can find the mean unfolding force of the first domain to unfold at that pulling speed. A similar process can be carried out for the second, third, fourth and fifth unfolding events and thus the dependence of the unfolding force on the event number at a given pulling speed can be plotted. Figure 4b shows this unfolding event number dependence of the unfolding force for experiment (squares) and simulation (circles).
Figure 4. (a) A typical mechanical unfolding force-extension dataset of \((C47S, C63S I27)_5\) by experiment (main curve) and simulation (inset). Both the experimental and simulated data were obtained with cantilevers of spring constant \(k_c = 50\) pN nm\(^{-1}\) and at a pulling speed of 700 nm s\(^{-1}\). The Monte Carlo data are obtained with an unfolded domain contour length of \(L_u = 28\) nm identical to that of the I27 domain (Carrion-Vazquez et al. 1999a; Brockwell et al. 2002). Other parameters in the simulations were: \(\alpha_{u,F} = 2 \times 10^{-3}\) s\(^{-1}\), persistence length of the unfolded domains \(p = 0.39\) nm and \(x_u = 0.29\) nm.

Before discussing the results of this analysis a comment must be made about the measured forces of the first unfolding events observed by experiment. The insets in figure 4b show the unfolding force–frequency histograms for the third event (total of 104 data points) and that of the first event (total of 104 data points). The histogram for the third event, which is typical of all but the first event, is narrow and well described by a single Gaussian distribution yielding a mean normalized unfolding force of 19.1% (data are normalized to the sum of all unfolding forces so that different datasets acquired on different days with different cantilevers can be combined to form a large enough dataset for statistical analysis). In the case of the first unfolding event, the distribution is not well fitted by a single Gaussian function, presumably due to domain–domain and domain–surface interactions, and is only well fitted by two Gaussian functions. The Gaussian centred on higher forces (19.9%) is taken as the actual domain unfolding force and the lower force (centred at 17.8%) presumably reflects spurious interactions of the unfolding protein with other domains and the gold surface. Despite this difficulty, it is clear from both Monte Carlo simulation and experiment that a minimum in the unfolding force is observed, in this case at the third unfolding event.
Figure 4. (Cont.) (b) Monte Carlo simulation (circles) and experimental data (squares) for unfolding forces of (C47S, C63S I27)$_5$ as a function of the unfolding event number # (see figure 1). Data are expressed as a fraction of the sum of the unfolding forces in order to combine many experimental datasets. Experimental data are expressed as a weighted mean ± weighted standard error of the mean. Inset shows relative force frequency histograms for #1 and #3 for all datasets (n = 104). Solid lines are fits to a single (#3) or a double (#1) Gaussian function. The points (×) and (∆) are the modes obtained from the fit to the distribution for #1.

What is the origin of this history effect in the mechanical unfolding of polyproteins? The reason that the minimum force in the unfolding sequence is not necessarily observed for the first unfolding event is that the number of folded domains remaining at any given time and the length of the domains already unfolded have competing effects on the unfolding force of the next domain to unfold. A decrease in the number of folded domains reduces the number of unfolding attempts at any given extension which decreases the unfolding probability. Thus, the measured mean unfolding force should rise monotonically as more domains unfold. However, as each domain is unfolded, the total length of unfolded polypeptide present increases, which increases the overall compliance (or reduces the effective spring constant) of the system. In a system with a higher compliance, $J$, the loading rate $df/dt = J^{-1} \nu$, where $\nu$ is velocity, is reduced, resulting in more thermally driven unfolding attempts per unit time at each extension, which would result in a gradual lowering of the unfolding force. The net result of these competing effects is the observed minimum in the unfolding force as a function of the unfolding event number.

The effect of the system compliance on the unfolding forces can be seen in figure 5. Both the cantilever spring constant and the length of the polypep-
Figure 5. Monte Carlo simulations showing the effect of varying (a) the unfolded contour length $L_u$ and (b) the AFM cantilever spring constant $k_c$ upon the observed unfolding forces at each event number. In (a) $k_c = 50 \text{ pN nm}^{-1}$ and in (b) $L_u = 28 \text{ nm}$.

tide chain released as each domain unfolds contribute to the total compliance of the mechanical system, and therefore both affect the loading rate in the experiment.
An increase in the length of the unfolded domain to 45 nm from the value of 28 nm (I27) causes the minimum in the unfolding force to become more apparent, because in the early events the fractional contribution to the change in compliance by unfolding a larger protein is greater (figure 5a). This effect is more clearly seen when a very stiff cantilever is used, since the contribution of the polypeptide chain length is a more significant factor than when a very compliant cantilever with low spring constant is employed (figure 5b). Our observations have important implications for understanding the mechanical properties of heteropolymers that have evolved naturally to resist force in vivo. The passive effect of unstructured polymers acting as an ‘entropic spring’ is well known (for instance the PEVK domain of titin (Linke et al. 1998) and the selectin cell–surface carbohydrate interaction (Fritz et al. 1998)). We have now shown that both the superstructure, or scaffold, in which the polymer is held and the number and length of unfolded domains influence the mechanical resistance of the remaining folded domains. Thus, effects such as the compliance of the surrounding tissue and the lengths of unstructured regions will play a key role in tailoring the mechanical resistance of folded domains in polyproteins. These observations add another level of complexity to any valid description of the mechanical properties of naturally occurring polyproteins and reveal the wide range of parameters available in biology for tuning the resistance of proteins to applied force for specific mechanical roles.

5. Summary and outlook

Mechanical unfolding studies of I27 from several laboratories indicate that the number and geometry of interstrand hydrogen bonds and a transition state unusually close to the native state maximize the mechanical resistance of the protein. Steered molecular-dynamics simulations have suggested that the mechanical stability of β-sheet proteins depends critically on the topology of the protein. Proteins with parallel N- and C-terminal strands exhibit the largest mechanical unfolding forces because all of the interstrand hydrogen bonds must be simultaneously broken for the protein to unfold (Lu & Schulten 2000). Proteins with anti-parallel terminal β-strands unfold at relatively low forces, possibly because the force is applied parallel to the hydrogen bond and results in the sequential ‘zipper-like’ rupture of these bonds with relatively low force (Rohs et al. 1999). This parallel β-strand secondary structure and hydrogen-bond clamp region give I27 the largest mechanical strength of any protein studied to date (ca. 200 pN for the wild-type protein). In comparison tenascin (FNIII domains), barnase, T4 lysozyme, the C2 domain of synaptotagmin I and spectrin unfold at 140 pN (Oberhauser et al. 1998), 65 pN (Best et al. 2001), 64 pN (Yang et al. 2000), 60 pN (Carrion-Vazquez et al. 2000) and 30 pN (Rief et al. 1999), respectively. Calmodulin unfolded at too small a force to be measured (Carrion-Vazquez et al. 2000). These data suggest therefore that proteins with β-sheet secondary structure are mechanically most stable, while α-helical proteins are relatively mechanically unstable. Proteins with mixed α/β topologies fall in between these two extremes.

The data we have presented here firstly support the conclusions of other researchers that mechanical resistance is mainly a locally endowed feature; destabilization of the core of I27 has little effect upon the mechanical sensitivity (\(F_{\text{xu}}\)) of the protein. In addition we have shown that the mechanical and chemical unfolding processes are
different insofar as the intrinsic unfolding rate constants are significantly different and that altering the stability of the native state by mutagenesis or addition of sodium sulphate affects the intrinsic unfolding rate constants of the I27 mutant studied here differently. An unexpected dependence of the unfolding force of a given unfolding event on its position in the sequence of unfolding events of the entire concatamer was observed. This is due to the effect of the number of folded domains remaining to be unfolded changing and the increased compliance of the mechanical system as domains unfold. The implication of these observations is that both the length of the unfolded domain and the compliance of the surrounding tissue will affect the mechanical resistance of a folded protein in its biological context. The mechanical resistance of a protein domain is modulated by these effects and therefore cannot be simply regarded as a property endowed by aspects of local secondary structure.

One particularly important aspect of the mechanical unfolding experiment is that the direction in which the force is applied can, in principle at least, be controlled. In contrast to other unfolding experiments, in which a low-molecular-weight denaturant (chemical unfolding), temperature or pH is used, and the unfolding reaction coordinate with respect to protein structure coordinates is not under experimental control. We are currently conducting studies in which a protein is mechanically unfolded using force applied in well-defined and different directions in separate experiments. Our preliminary results indicate that the unfolding forces observed vary more than 10-fold, depending on the direction of the applied force relative to a hydrogen-bond clamp. Such experiments open the way for a detailed mapping of the mechanical unfolding energy landscape and an extensive comparison of unfolding experiments with molecular-dynamics simulations.

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References


Mechanically unfolding proteins


**Discussion**

W. T. Coffey (*School of Engineering, Trinity College Dublin, Ireland*). You use transition state theory to describe the reaction rate which is based on equilibrium considerations. Kramers (1940) made a major improvement in transition-state theory by taking into account the coupling to the heat bath. This allowed him to connect the reaction rate to the Langevin equation describing the motion of a reacting particle, thus non-equilibrium effects could be included in the reaction rate. Moreover, the dependence of the reaction rate on the parameters of the Langevin equation, inertia, friction, etc., could be determined. Subsequently, this theory was generalized to a reacting system of n degrees of freedom by Langer (1969), which *inter alia* constitutes a general theory of the decay of metastable states. It seems to me that Langer’s treatment would provide a useful basis for the theoretical discussion of your problem.

D. A. Smith. We are in fact using the results of the Kramers calculation for the rate of passage over a barrier, in the adiabatic limit, as discussed by Bell (1978) and Evans & Ritchie (1997). The effects of the applied force are to lower the barrier in a physically appealing manner, which we include, as well as to weakly modify the prefactor or ‘attempt frequency’, which (following Evans & Ritchie) we do not incorporate. This treatment can, in fact, be related to a Langevin equation and, as derived by Kramers, is valid in the limit of strong damping. Langer’s calculation for n degrees of freedom may in fact be relevant, but without substantial information...
about the nature of other degrees of freedom (which undoubtedly are there but are probably quite fast and not accessible in our experiments) analysis in this manner is not possible.

E. Sackmann (Faculty of Physics, Technical University of Munich, Garching, Germany). You appear to be able to measure the unfolding of a protein under applied force with exquisite accuracy. Could similar techniques be used to measure the interaction potential between two particles: two proteins for example?

D. A. Smith. That is indeed the case, although current instrumentation has a practical force resolution (due to Brownian motion of the cantilever) of \( \text{ca.} \) 15 pN, which clearly limits the range of ‘particles’ that one might be able to study. However, developments in instrumentation (see, for example, Aoki et al. 1997) permit at least an order of magnitude increase in force sensitivity, opening the possibility for experiments to study the interaction potentials in a wider range of cases.

B. U. Felderhof (RWTH, Aachen, Germany). The discussion in terms of energy landscape is only valid in the adiabatic limit. It seems to me that the dependence on the rate at which you are pulling, or on frequency in an oscillating experiment, necessarily involves hydrodynamic effects due to friction with the ambient fluid.

D. A. Smith. You are correct that the Kramers calculation assumes adiabaticity, i.e. the pulling speed is slow compared with the rate at which the pulled molecule explores its energy landscape (internal degrees of freedom). This treatment is incorrect at pulling speeds well beyond AFM capabilities. Hydrodynamic friction effects are quite small. For example, the friction due to the cantilever at a pulling speed of 500 nm s\(^{-1}\) is only a few pN (assuming Stokes drag), while that on the individual domains is much smaller and negligible on the scale of the measured unfolding forces (of the order of 100–200 pN).

M. Maaloum (Institut Charles Sadron, Strasbourg, France). The force values you measure depend on the precision of the spring constant. How do you calibrate the cantilever? Do you stretch the same molecule or different molecules in each experiment? Is the force profile reversible?

D. A. Smith. The spring constant of each cantilever was calibrated under PBS using the thermal noise method (Florin et al. 1995) and was typically found to be \( \text{ca.} \) 51 ± 5 pN nm\(^{-1}\). In general, different molecules are stretched each time the experiment is performed, mainly due to thermal drift of the sample below the AFM tip. However, with care it is perfectly possible to pull the same molecule repeatedly. The force profile is repeatable but not reversible. This is because the protein will not refold under even the slightest applied force. Thus, the tip with the protein attached must be returned to the substrate to release all tension in the system before refolding will occur (see, for example, Carrion-Vazquez et al. 1999a).

S. Titmuss (Physical and Theoretical Chemistry Laboratory, University of Oxford, Oxford, UK). You associate the sawtooth force profiles with the unfolding of a single pentameric construct; presumably sometimes you pick up more than one pentamer. How do you distinguish between the case of single and multiple constructs on the tip?

D. A. Smith. If multiple proteins are picked up and extended together then it is very clear because the extension between the sawtooth peaks does not correlate to the expected extension due to the length of an unfolded domain.

D. S. F. Crothers (Department of Applied Mathematics and Theoretical Physics, Queen’s University, Belfast, UK). In your Monte Carlo simulations, which variables are randomized?

D. A. Smith. We calculated a transition probability as a function of applied extension (using the Kramers result for the first passage time as a function of force applied to a potential). A random number was then chosen; if this number was less than the calculated transition probability then the event was accepted and unfolding occurred. Our Monte Carlo simulations are based on the treatment by Rief et al. (1998). The intrinsic unfolding rate constant and the distance to the transition state from the native state are used as fit parameters to achieve the best fit to the experimental data (see Brockwell et al. (2002) for details).

P. Bartlett (School of Chemistry, University of Bristol, UK). Have you thought about the possibility of doing the experiment at constant force rather than at constant rate of pulling? It might be possible, for instance, to access the significance of fluctuations more readily by sitting at the top of the free energy barrier.

D. A. Smith. That is a very nice experiment which we have not done but has been done in Paul Hansma’s laboratory (Oberhauser et al. 2001). One can observe the rate of occurrence of unfolding events and obtain the unfolding rate constants at different applied loads. In principle one could poise the system close to the transition state and study the effect of fluctuations on the unfolding rate constant. However, the dominant process could be the Brownian motion of the cantilever in a standard AFM (see my earlier response to Dr Sackmann).

Additional references

Kramers, H. A. 1940 Physica 7, 284.