

## Mechanical Unfolding of an Ankyrin Repeat Protein

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**ABSTRACT** Ankyrin repeat proteins comprise tandem arrays of a 33-residue, predominantly  $\alpha$ -helical motif that stacks roughly linearly to produce elongated and superhelical structures. They function as scaffolds mediating a diverse range of protein-protein interactions, and some have been proposed to play a role in mechanical signal transduction processes in the cell. Here we use atomic force microscopy and molecular-dynamics simulations to investigate the natural 7-ankyrin repeat protein gankyrin. We find that gankyrin unfolds under force via multiple distinct pathways. The reactions do not proceed in a cooperative manner, nor do they always involve fully stepwise unfolding of one repeat at a time. The peeling away of half an ankyrin repeat, or one or more ankyrin repeats, occurs at low forces; however, intermediate species are formed that are resistant to high forces, and the simulations indicate that in some instances they are stabilized by nonnative interactions. The unfolding of individual ankyrin repeats generates a refolding force, a feature that may be more easily detected in these proteins than in globular proteins because the refolding of a repeat involves a short contraction distance and incurs a low entropic cost. We discuss the origins of the differences between the force- and chemical-induced unfolding pathways of ankyrin repeat proteins, as well as the differences between the mechanics of natural occurring ankyrin repeat proteins and those of designed consensus ankyrin repeat and globular proteins.

### INTRODUCTION

Repeat proteins comprise tandem arrays of small structural motifs (30–50 residues) that pack in a roughly linear fashion to produce elongated, often superhelical architectures. They are composed of short-range interactions between residues either within a repeat or in adjacent repeats, and as such they contrast with globular proteins, which are stabilized by many sequence-distant interactions that frequently result in complex topologies. Each ankyrin repeat forms a  $\beta$ -turn followed by two antiparallel  $\alpha$ -helices and a loop. The folding and stability of several ankyrin repeat proteins have been studied in detail, and these proteins have been found to possess certain features that distinguish them from the more commonly studied globular proteins. These features arise from the symmetry inherent in their structures and the absence of long-range interactions (1). In particular, it is relatively easy to dissect their biophysical properties, and consequently they are highly amenable to redesign of their thermodynamic stability, folding mechanisms, and molecular recognition (2–4). The mechanics of ankyrin repeat proteins remains largely unknown, in contrast to globular proteins, whose mechanical properties have been examined quite well through the use of single-molecule force spectroscopy measurements and steered molecular dynamics (MD) (5–11).

A number of ankyrin repeat proteins have been proposed to mediate mechanotransduction in a variety of different functional settings (12–14). For example, ankyrin repeat domains may act as an elastic element allowing the opening and closing

of ion channels in response to stimuli (15). Many ankyrin repeat proteins function as scaffolds in protein-protein interactions, which may require other specific mechanical properties. In an effort to understand the molecular basis of these putative mechanical functions, we investigated the behavior of small ankyrin repeat proteins, the folding and stability of which we previously studied in solution. We characterized the mechanical unfolding of the 7-ankyrin repeat protein gankyrin using atomic force microscopy (AFM) and MD simulations. Gankyrin is a recently identified oncoprotein that is involved in multiple protein-protein interactions and is a negative regulator of two principal tumor suppressors: p53 and pRB (16). MD simulations reveal multiple distinct unfolding pathways of gankyrin, all of which are noncooperative and proceed via partly folded intermediate species, in some cases stabilized by nonnative interactions. The intermediates are long-lived and resistant to high forces. This broad landscape of extension pathways observed for gankyrin is distinct from the more homogeneous unfolding of the consensus-designed 5-ankyrin repeat protein NI3C. The AFM data are consistent with the high-resolution picture obtained from the simulations: gankyrin unfolds noncooperatively, with the peeling away of half a repeat, or one or more repeats at a time at low force, and intermediates are populated in some traces that unfold only at high force. Finally, the unfolding of the individual ankyrin repeats is shown to generate a refolding force, and we discuss the possible structural basis for this phenomenon. We compare the behavior of ankyrin repeat protein unfolding under force and in solution, and the mechanical properties of natural versus consensus-designed ankyrin repeat proteins, and we discuss the possible physical origins of the differences observed.

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## MATERIALS AND METHODS

### Construction and characterization of I27GKN polyprotein

Our construct, I27GKN, was made using multiple copies of the I27 domain of titin as a scaffold. The I27 polyprotein vector was a kind gift from J. Clarke (University of Cambridge, Cambridge, UK) (17). I27GKN consists of one gankyrin flanked by three and four I27 domains at the N- and C-terminus, respectively. The gankyrin construct was a kind gift from A. Wilkinson (University of York, York, UK). Protein expression and purification were performed as described previously (17) except that a gel filtration step was added. The protein was flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Analytical gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis were regularly used before and after freezing to confirm the absence of proteolysis and aggregation.

### AFM

Several different methods were used to immobilize the protein molecules. AFM measurements were made using custom-built AFM instruments equipped with an AFM detector head (Veeco Metrology Group, Santa Barbara, CA) and high-resolution piezoelectric stages (Physik Instrumente, Irvine, CA) equipped with position sensors (vertical resolution: 0.1 nm). The spring constant of each MLCT-AUHW microcantilever or Bio-Lever cantilever (Veeco) was calibrated by using the energy equipartition theorem. Molecules were picked up for stretching measurements by gently touching the substrate with the AFM tip, using the nonspecific adsorption of the construct to the tip. Force-extension measurements were performed at a pulling speed of 50 nm/s at room temperature ( $20\text{--}25^{\circ}\text{C}$ ) and in solution using different buffers (50 mM PBS, pH 7.5; 50 mM Tris-HCl, pH 8, 1 M NaCl; 50 mM Tris-HCl, pH 8, 150 mM NaCl; 50 mM Tris-HCl, pH 8, 700 mM NaCl, 200 mM imidazole, 1 mM DTT, pH 8; and 50 mM HEPES, pH 8). All of the buffers produced the same kinds of traces. Traces were fitted to a worm-like chain (WLC) model of polymer elasticity to obtain the contour length and the persistence length.

### MD simulations

MD simulations were performed using the CHARMM program (18). Simulations were performed using two different implicit solvent models: effective energy function 1 (EEF1) (19) and fast analytical continuum treatment of solvation (FACTS) (20). Implicit solvents were preferred to explicit ones because an atomistic representation of even a single hydration layer of an extended protein conformation would require an immense computational effort; moreover, implicit solvents relax instantaneously, which reduces artifacts when the protein is pulled fast.

Great care was taken to assess potential artifacts of the simulations and the robustness of the results obtained. For this reason, the constructs were unfolded both by elongating springs attached to both ends at constant velocity or by applying a constant force. An equal force was applied to the N-terminal main-chain nitrogen and to the C-terminal carbonyl carbon, along the vector joining the atoms and in the direction of increasing distances.

For the constant-velocity experiments, pulling speeds of 0.5, 0.2, 0.1, and 0.05  $\text{\AA}/\text{ps}$ , and elastic constants of 10, 20, and 100 pN/ $\text{\AA}$  were used. For the constant-force simulations, forces of 40, 50, 80, 90, 100, 125, 200, 300, 400, 500, and 600 pN were used. We screened a range of different forces, speeds, and elastic constants to find the regime closest to the experimental conditions where we could obtain a detailed picture of the unfolding process on a reasonable timescale. At forces above 300 pN, the protein unfolded without showing any distinct step, and below 125 pN we did not observe complete unfolding. Simulations were performed for 30–100 ns, depending on how long it took for the protein to unfold fully. Forty constant-force simulations were performed on the gankyrin monomer using EEF1, and 33 were performed using FACTS; 17 and 20 of these simulations, respectively, were performed at the optimal force of 125 pN. Twenty simulations

with the N13C crystal structure 2QYJ were done using FACTS at 125 pN. Five constant-velocity simulations were performed on the gankyrin monomer using EEF1, and two were done using FACTS. Twenty constant-velocity simulations (using EEF1 only) were performed on the I27GKN polyprotein.

Initial conformations were generated using the crystal structures 1QYM for Gankyrin, 2QYJ for the consensus ankyrin N13C, and 1TIT for I27. The polyprotein I27GKN was built from the coordinates of the individual proteins using Swiss-PDB Viewer (21) and Chimera software (22). Langevin dynamics at 300 K with a friction coefficient of 1 ps and an integration time step of 2 fs was used.

## RESULTS

### MD simulations of mechanical unfolding

We simulated the mechanical unfolding of three proteins: the crystal structure of gankyrin (1QYM(23)), the crystal structure of the consensus ankyrin N13C (2QYJ (24)), and the I27GKN polyprotein formed by seven I27 domains and one gankyrin protein (see the [Supporting Material](#)). Two different implicit solvent approaches were used for the simulations of the gankyrin monomer (see [Materials and Methods](#)), and they produced the same overall picture. The gankyrin monomer was found to reach a stable conformation after 2 ns of equilibration using either solvent model, and the  $C\alpha$  root mean-square deviation from the crystal structure was always  $<2 \text{\AA}$ . The equilibrated protein differed slightly from the crystal structure in the partial straightening of the curvature of the repeat stack and in the weakening of the packing between repeats. The terminal repeats also changed their orientation slightly relative to the internal repeats when compared with the crystal structure.

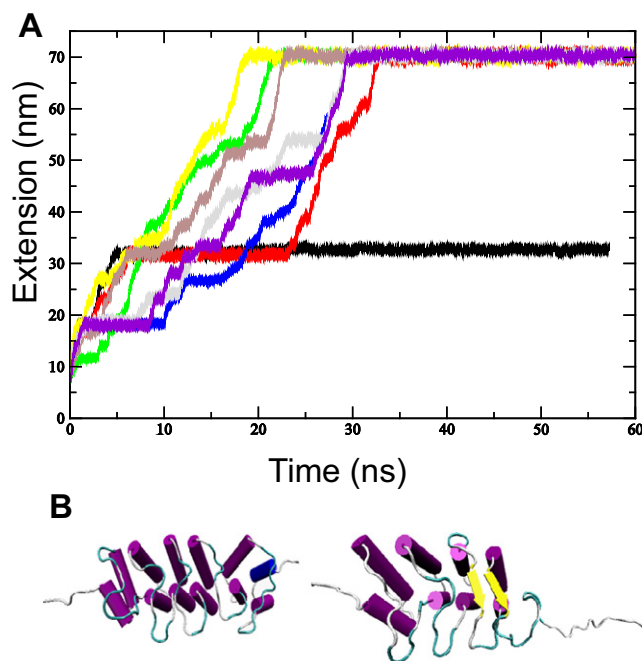
Below, we present the results obtained using FACTS implicit solvent. A larger number of simulations were performed with this solvent, and under constant force only (for the results of the constant-velocity simulations, see the [Supporting Material](#)). Our conclusions regarding the mechanisms and differences between the species studied here apply to both the force fields and pulling method employed, and thus underline the robust properties of the constructs.

### Constant-force simulations of the gankyrin monomer

It is fairly difficult to impose constant force in single-molecule force microscopy experiments, but it is straightforward in simulations (25). We performed 20 simulations of gankyrin 1QYM (seven repeats) and the consensus ankyrin 2QYJ (five repeats) using a 125 pN constant force. At this force, unfolding was noncooperative, displaying multiple phases and without an initial lag phase. The phases differed from one another in both end-to-end distance and lifetime, and they were also different in the different simulations. Unfolding started at the C-terminus in 19 of the 20 simulations and progressed to the N-terminus. In those simulations in which an intermediate was populated, the unfolding started at the C-terminus and progressed to the N-terminus

up to the repeat that underwent rearrangement. Then unfolding of the most N-terminal repeat occurred and progressed from the N-terminus to the C-terminus. The most frequently observed intermediates resulted from the unfolding of either one or three C-terminal ankyrin repeats together with the reorientation of the remaining helices. Analysis of the trajectories showed that before the helices could unfold, they first had to align with the axis of the pulling vector. The secondary structure timeline analysis indicated that early disruption of the hydrogen bonds in the  $\beta$ -turns at the bases of the long loops connecting adjacent ankyrin repeats allowed a greater degree of freedom for the helices to explore nonnative contacts, including the formation of  $\beta$ -sheet structures (Fig. 1 B). Seven of the 20 simulations showed long-lived intermediates (remaining at a constant length for more than one-third of the total simulation time).

Gankyrin underwent partial unfolding at low forces involving the extension of the interrepeat packing and the



**FIGURE 1** Constant-force simulations of the mechanical unfolding of gankyrin (IQYM) at 125 pN using FACTS implicit solvent. (A) Plot of extension versus time for a number of representative simulations. Unfolding occurs in a noncooperative manner, with multiple phases lasting for different periods of time. In some of the runs, the protein is trapped in a force-resistant intermediate state (red, blue, and violet traces) and in one run it does not reach the unfolded state within the duration of the simulation (black trace) (58 ns). Some other traces (in yellow and green) unfold very quickly without any long-lived intermediate state. (B) Left: Structure of the intermediate formed in the blue trajectory in panel A. The intermediate results from the unfolding of the most C-terminal repeat and part of the adjacent repeat. There is some disruption of the native packing in the structured regions, and nonnative contacts begin to form. Right: Structure of the long-lived intermediate formed in the black trajectory in panel A. The intermediate results from the unfolding of the three C-terminal ankyrin repeats and is stabilized by the formation of a nonnative parallel  $\beta$ -sheet formed between the long loops connecting two adjacent repeats (in yellow).

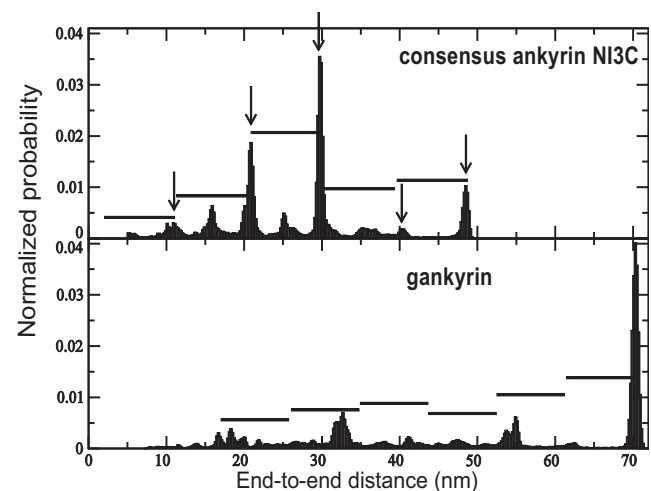
unfolding of a terminal repeat, most frequently the C-terminal one. This behavior contrasts with that of mechanically resistant proteins, such as I27, protein L, and ubiquitin, which typically unfold in an all-or-none fashion or via a single intermediate only.

The distribution of extension lengths for gankyrin differed from that observed in the consensus ankyrin protein NI3C (Fig. 2). For NI3C, the most frequent extensions (10 nm, 20 nm, and 30 nm) were consistent with the unfolding of individual ankyrin repeats (10 nm each). In contrast, for gankyrin the distribution of extensions was much broader than the consensus. This means that gankyrin unfolds through less clearly defined steps, which for the consensus ankyrin correspond to the cooperative unfolding of individual repeats.

To further investigate the unfolding mechanism of the two different ankyrin repeat proteins, we analyzed the traces of the extension versus time and assumed that a well-defined metastable state was populated if the extension was constant (within 5 Å fluctuations) for at least 0.6 ns. In Fig. 3 we plot the distribution of the extension increments ( $\Delta Lc$ ) between consecutive metastable states. The most probable increments corresponded to the unfolding of half of one repeat ( $\sim 5$  nm) and of one repeat ( $\sim 10$  nm) for both NI3C and gankyrin. However, gankyrin showed frequent extensions ranging from 15 nm (1.5 repeats) to 37 nm (3.5 repeats), and these were much less frequent for NI3C.

### AFM of I27GKN

We used our polyprotein, I27GKN (gankyrin flanked by three I27 domains at its N-terminus and four I27 domains



**FIGURE 2** Representation of the distribution of extension lengths in the constant-force simulations. The upper panel shows the consensus ankyrin repeat NI3C, and the bottom panel shows gankyrin. Bars indicate the spacing corresponding to the length of one unfolded ankyrin repeat, and arrows indicate intermediates in which an integer number of domains is fully extended. Although they are more rare, conformations in which half a repeat is extended also exist. For gankyrin, the peaks are broad and their periodicity is not clearly related to the extension of individual domains.

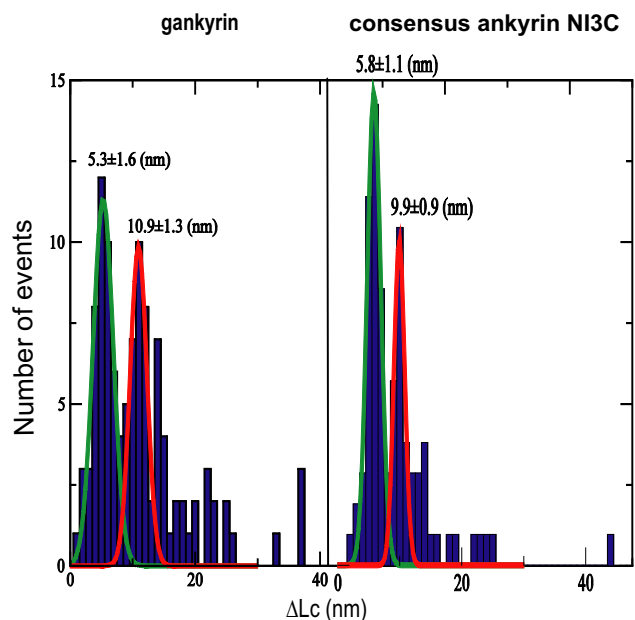


FIGURE 3 Histograms of the extension length increments ( $\Delta Lc$ ) between consecutive metastable states for gankyrin (*left panel*) and N13C (*right panel*). Both proteins show two major increments, corresponding to 5–7.5 nm (half of an ankyrin repeat) and 10–12.5 nm (one ankyrin repeat). However, gankyrin shows other high-frequency increments ranging from ~12.5 nm to ~36 nm.

at its C-terminus), as a reference to subsequently identify the mechanical unfolding behavior of gankyrin monomers and to obtain a distribution of the increments in contour length upon stretching (Fig. 4). We obtained measurements using several different buffers and immobilization methods (at either the N- or C-terminus of the polyprotein) to rule out artifacts associated with these variables (see [Supporting Material](#)). All conditions produced similar results. We also tested a buffer containing a high concentration of imidazole to check whether the high force peaks observed in some

traces were due to interactions of the histidine residues in the gankyrin protein interacting with the NTA-functionalized glass surface. The presence of imidazole did not affect the traces obtained, which ruled out that possibility but did not exclude a nonspecific interaction with the surface. These traces were discarded for the quantitative analysis.

We analyzed only those force-extension curves that clearly captured the mechanical fingerprint of the unfolding of four or more I27 domains of the I27GKN polyprotein with an initial extension length corresponding to the stretching of gankyrin (~82 nm) plus the whole folded construct ( $n \times 4.5$  nm;  $n$  = number of I27 modules picked up by the AFM tip), and discarded those traces with changes in the persistence length between force peaks (in previous constant-velocity simulations, gankyrin unfolded first, followed by the I27 domains; see [Supporting Material](#)). Traces with four I27-domain unfolding events were also included in the analysis if they captured full-length gankyrin. This allowed us to unequivocally identify the recordings that were obtained on single molecules as compared to recordings obtained on multimolecular structures (only 1% of the traces recorded were used to build the extension histogram in Fig. 4). We observed that upon stretching, the whole of gankyrin (or parts of it) unfolded before any I27 domains unfolded. In these traces, force peaks ranging from 40 pN to 150 pN were recorded during the initial phase of stretching (Fig. 4 A), which we attribute to the unfolding of gankyrin. These force peaks were followed by regular force peaks at ~200 pN, spaced by ~27–29 nm as determined by the WLC fits (26), which we attribute to the unfolding of I27 domains. Fittings of these unfolding peaks with a WLC model of polymer elasticity provided the distribution of the increments in contour length (Fig. 4 D). In this histogram, we observe that the most probable increments in contour length correspond to the unfolding of half or one ankyrin repeat. However, the unfolding of two or more repeats at

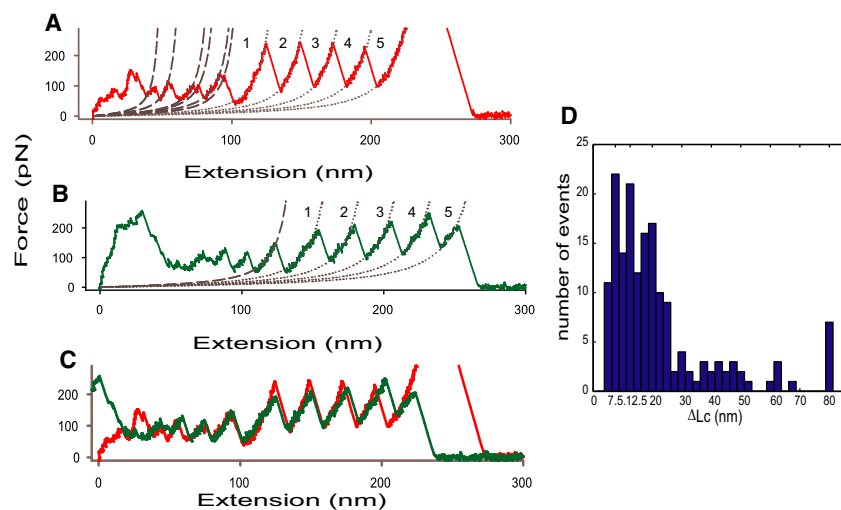


FIGURE 4 Histogram of the extension length increments ( $\Delta Lc$ ) of the unfolding peaks of gankyrin in the polyprotein I27GKN measured by AFM. Forty-two force-extension curves, containing a total of 170 unfolding peaks of the gankyrin portion of the construct, were fitted to a WLC model of polymer elasticity and the  $\Delta Lc$  values were extracted. (A) Force-extension plot containing the unfolding of five I27 domains and gankyrin. The unfolding traces were fitted to a WLC model of polymer elasticity and the  $\Delta Lc$  values were extracted. The fittings for the I27 domains are represented by dotted lines. The fittings for gankyrin are represented by dashed lines. (B) A different trace containing the unfolding of five I27 domains and gankyrin. (C) Superimposition of traces A and B, showing a very accurate matching. (D) Histogram for the  $\Delta Lc$  values. The most probable events correspond to the unfolding of one or a half ankyrin repeat. However, extensions corresponding to the unfolding of two or more repeats are also highly represented.



a time was also frequently observed. A small subset of traces did not produce detectable force peaks and were plotted as extensions of  $\sim 80$  nm, which may correspond to gankyrin being already unfolded before stretching.

### AFM of the gankyrin monomer

We performed experiments using the gankyrin monomer to capture the refolding of gankyrin, which would involve a complicated procedure in a long polypeptide chain such as the construct I27GKN. The molecules were adsorbed, at a low surface density, to a clean glass substrate and picked up by the AFM tip. We limited the stretching distance below the contour length of the fully unfolded gankyrin monomer to avoid its detachment from the AFM tip. In this way, we were able to perform multiple stretch-and-relax cycles on the same molecule and examine its refolding behavior. Successive force-extension curves are shown in Fig. 5. The force peaks obtained in the second stretching cycle overlap with the original unfolding force peaks. Of note, in the third stretching cycle there was a single  $\sim 200$  pN unfolding force peak, and in the fourth stretching cycle the force peaks were similar to the original force peaks. In all four cycles, refolding was found to generate a force of  $\sim 15$  pN (measured as the amplitude from the baseline to the unfolding peak). Similar refolding force peaks were previously observed in 12- and 24-ankyrin repeat proteins (27).

## DISCUSSION

### Mechanical unfolding of gankyrin occurs via multiple distinct pathways and intermediate species

AFM experiments using 24- and 12-repeat fragments of ankyrin-B showed that the ankyrin repeats can unfold one or two at a time under low forces of  $<50$  pN (27) after an initial hook-like, high-force unfolding peak. This feature was also presented in a recently proposed mathematical model for the mechanical unfolding of ankyrin repeats (28). This range of forces is similar to that observed by Li et al. (29), who found that a consensus-designed, 8-ankyrin repeat protein unfolded in a fully stepwise manner one repeat at a time. Simulations of 12- and 24-ankyrin repeats also showed the unfolding and refolding of individual repeats after stretching of the superhelical stack (30). Our study, which combines experiment and simulation, reveals a number of new details about the mechanical unfolding of ankyrin repeat proteins, in particular concerning the structures of intermediate species. The results point to multiple pathways of mechanical unfolding of gankyrin and suggest a rugged and complex energy landscape. The protein does not unfold in a single cooperative step under force, nor does it always unfold in a fully stepwise manner one repeat at a time. Instead, gankyrin proceeds through different intermediate species resulting from the unfolding of half a repeat,

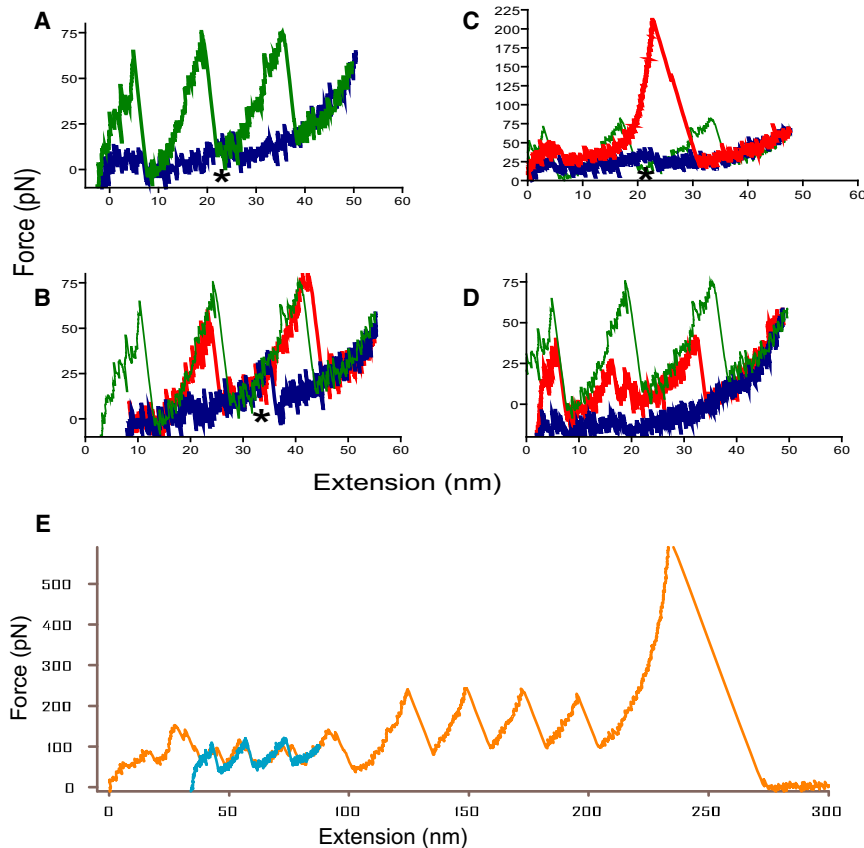


FIGURE 5 Examples of unfolding and refolding force-extension relationships measured by AFM on the same gankyrin molecule in a cyclic measurement. (A) The first unfolding cycle (green) and refolding (blue) captured three clear  $\sim 60$ – $80$  pN unfolding peaks at  $\sim 5$ , 20, and 40 nm of the extension, and a  $\sim 15$  pN refolding peak (black star). (B) The molecule was lifted by 5 nm away from the substrate and stretched and relaxed again. The two unfolding force peaks (shown in red) overlap with the original unfolding force peaks (shown in green), and the relaxing trace (blue) captured one refolding force peak (black star). (C) The next stretching cycle (red) captured a single  $\sim 200$  pN unfolding force peak and the relaxing trace captured a small refolding event (black star). (D) A subsequent pulling cycle revealed  $\sim 50$  pN unfolding force peaks similar to those measured during the first unfolding cycle (in panel A). (E) Superimposition of force-extension curves of the gankyrin monomer and the I27GKN construct. Of note, gankyrin is shifted to the right to account for the extension of the folded polypeptide, showing a very accurate matching.

or one or more repeats at low force. Some of the long-lived and force-resistant intermediates observed were stabilized by nonnative interactions. Of note, a high-force unfolding peak of 200 pN was observed in the third stretching cycle of the gankyrin monomer, which could be rationalized by the long-lived intermediates observed in the simulations of forced unfolding of gankyrin. Alternatively, it could correspond to the unfolding of a misfolded (nonnatively folded) species formed in the previous relaxation cycle. This conjecture is supported by the refolding trace in Fig. 5 B, which captured an unusually high-force refolding peak. Such a robust refolding event is suggestive of the formation of a structure that is more resistant to mechanical unfolding than the native fold. In addition, the 200 pN unfolding peak in Fig. 5 C is not followed by other unfolding events that would have been expected to complete the unfolding of the putative intermediate. However, since some of the unfolding intermediates observed in the simulations were stabilized by nonnative interactions, it is possible that the refolding and unfolding intermediates are similar in nature.

### Mechanical versus solution unfolding mechanisms of ankyrin repeat proteins

We can compare the mechanical unfolding of gankyrin with the results of numerous solution unfolding studies of ankyrin repeat proteins. Myotrophin (4), gankyrin (R.D. Hutton, A.R. Lowe, and L.S. Itzhaki, unpublished results), and D34 (31) unfold in solution via multiple pathways under kinetic conditions, whereas only a single pathway has been detected for p16 (32) and Notch ankyrin domain (33). The rate-limiting transition states for all of these reactions, with the exception of Notch, have polarized structures with either N-terminal or C-terminal repeats folded. For consensus-designed ankyrin repeats, equilibrium and kinetic intermediates were observed that comprised a subset of folded repeats and followed an Ising-like folding model, and the formation of a single repeat was indicated as the rate-limiting step in the folding reactions (34). Whereas smaller natural ankyrin repeat proteins (comprising three or four repeats) (35–37) do not accumulate intermediates upon solution unfolding under kinetic conditions, larger ankyrin repeat proteins do (38–41), and gankyrin unfolds via a high-energy intermediate that can only be detected indirectly. However, in contrast to force-induced unfolding intermediates, there is no evidence for substantial nonnative structure in any of these intermediates or in the unfolding transition states (within the limits of the protein engineering approach used).

In mechanical unfolding, the force is applied in a single direction and usually to the ends of the molecule, whereas chemical denaturants act on the protein globally; therefore, the resulting distortion of the native structure might be expected to differ between the two reactions. A number of comparative studies have indeed shown that the chemical- and force-induced unfolding pathways are different for

gankyrin (42–44). Further, the consensus 8-ankyrin repeat protein was found to unfold under force by peeling away one repeat at a time (29), in contrast to the more cooperative kinetic unfolding mechanism that was observed in solution involving only one or two intermediate species (34). Another difference between chemical denaturant and force is that, when the force is applied and regions of the protein unfold, partly folded intermediates can become populated because the applied force is transiently reduced as it is dissipated by those regions that are unfolded and stretched. This type of behavior was observed in a study of I27, i.e., one strand unfolded before the rest of the protein (7). Thus, force-induced unfolding is more likely than chemical-induced unfolding to reveal partly folded species.

One property that does appear to be common to both the force- and chemical-induced unfolding reactions of gankyrin is the accessibility of more than one pathway. In different simulations, the mechanical unfolding proceeded via different types of intermediate species, and in the AFM experiments we likewise observed several different types of traces.

The refolding of ankyrin repeats generates a force. This was observed previously (27) and can be rationalized as follows: Repeat proteins appear to have a tendency to unfold under force in a stepwise manner. As a consequence of this autonomy of the individual repeats, the energy barrier for refolding may be lowered in two ways. The first is achieved by a templating effect of the already-folded repeats. Second, the local nature of the contacts within a repeat and the short contraction distance involved will result in a lower entropic cost for folding repeats compared to globular proteins. The lower contact order does not appear to give rise to faster folding rates in solution, but this may reflect the different folding mechanism in solution versus that in an AFM experiment. In contrast to repeat proteins, partly structured species are much less stable in globular proteins, and therefore many long-range interactions involving the whole polypeptide chain, including those of the tethered termini in an AFM experiment, have to be made before refolding can occur.

Finally, it is possible that natural and consensus-designed ankyrin repeat proteins respond somewhat differently to force. The latter proteins have a much more extensive and regular hydrogen-bonding network in the  $\beta$ -turns adjacent to the long loops (45) and consequently a much higher thermodynamic stability (34). This feature could explain the more brittle and homogeneous behavior of the designed ankyrin repeat protein under force characterized by a very regular sawtooth pattern in the AFM traces (29) and a narrow population of unfolding contour length units ( $\Delta L_c$ ) compared to gankyrin. We speculate that the weaker hydrogen bonding in the loops and the longer loop lengths of natural ankyrin repeat proteins (particularly the TRP channels, which may have a mechanical function) might also facilitate the formation of nonnative interactions. In summary, we find first that gankyrin is weak in that one or more repeats can unfold at low forces. However, the nonnative intermediates

observed in the simulations are resistant to high force, and these could act as an important safety mechanism against complete unfolding despite the lack of specific force-bearing native interactions. Second, gankyrin mechanically unfolds following multiple extension pathways, having more low-energy unfolding pathways than the consensus ankyrins. Of interest, unfolding started at the C-terminal repeat in 95% of the simulations. Third, the unfolding of gankyrin, like that of ankyrin-B, can generate a refolding force that may be common to all ankyrins and relevant for functions in the cell when an elastic component is required.

The behavior of gankyrin contrasts with that of I27 and other mechanically strong proteins in which a specific subset of native interactions is clearly responsible for their stability. Because of the nonnative interactions observed in the intermediates, as well as the accessibility of multiple unfolding we speculate that the mechanical resistance of gankyrin is likely not related to specific interactions and thus may be relatively insensitive to single-site mutations. Instead, the presence of regularized interactions across the entire repeat array may be critical for the mechanical resistance of a repeat protein, and therefore multiple mutations that can affect this regularity (as achieved, for example, by consensus design) will be required to change its mechanical resistance.

## SUPPORTING MATERIAL

Four figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(09\)06136-0](http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)06136-0).

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