Fast and Forceful Refolding of Stretched α-Helical Solenoid Proteins

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ABSTRACT Anfinsen's thermodynamic hypothesis implies that proteins can encode for stretching through reversible loss of structure. However, large in vitro extensions of proteins that occur through a progressive unfolding of their domains typically dissipate a significant amount of energy, and therefore are not thermodynamically reversible. Some coiled-coil proteins have been found to stretch nearly reversibly, although their extension is typically limited to 2.5 times their folded length. Here, we report investigations on the mechanical properties of individual molecules of ankyrin-R, β-catenin, and clathrin, which are representative examples of over 800 predicted human proteins composed of tightly packed α-helical repeats (termed ANK, ARM, or HEAT repeats, respectively) that form spiral-shaped protein domains. Using atomic force spectroscopy, we find that these polypeptides possess unprecedented stretch ratios on the order of 10–15, exceeding that of other proteins studied so far, and their extension and relaxation occurs with minimal energy dissipation. Their sequence-encoded elasticity is governed by stepwise unfolding of small repeats, which upon relaxation of the stretching force rapidly and forcefully refold, minimizing the hysteresis between the stretching and relaxing parts of the cycle. Thus, we identify a new class of proteins that behave as highly reversible nanosprings that have the potential to function as mechanosensors in cells and as building blocks in springy nanostructures. Our physical view of the protein component of cells as being comprised of predominantly inextensible structural elements under tension may need revision to incorporate springs.

INTRODUCTION

Polypeptides provide numerous biological structures with the necessary strength and elasticity required for their functions (1–8), and have considerable potential as building blocks for bio-inspired elastomeric nanostructures and materials (1–5,9). In addition, spring-like proteinaceous structures are believed to mediate mechanosensation in metazoan organisms, which is critical for various physiological processes, such as hearing and regulation of blood pressure, as well as morphogenesis (10–13). For these reasons, how biomacromolecules and especially proteins respond to force is of considerable interest (14,15). Anfinsen's thermodynamic hypothesis (16) implies that, in principle, proteins can encode for their distortion due to stretching through reversible loss of structure. However, for most proteins, currently observed rates of refolding are either too slow and/or the refolding force itself too weak (17) to minimize hysteresis between stretching and relaxing, and therefore extreme extensions of proteins in vitro must dissipate a significant amount of energy. For example, tandem repeats of immunoglobulin (Ig)-like or fibronectin-like domains of titin, fibronectin, filamin, or tenascin are responsible for the passive elasticity of muscle and the extracellular matrix (5–8,18–22). When stretched in an atomic force microscope, they can be extended beyond their folded contour lengths by up to approximately seven times through mechanical unfolding of their domains (7). However, this unfolding-driven extension dissipates ~80–90% of the ~200–600 kcal/mol of energy required to fully stretch an Ig, fibronectin, or similar domain (1,2). Hence, titin-like proteins and their synthetic homologs (1) have been dubbed ''shock absorbers'' (1,2), although it is unlikely that significant unfolding of titin domains occurs in muscle under physiologically relevant strains (6).

On the other hand, coiled-coil proteins, which are components of motor proteins such as myosins and kinesins (23), and of keratins, through which they enable the elasticity of skin and hair (2), can be stretched in a nearly reversible manner by up to 2.5 times their initial length. Upon relaxation, they readily refold against a significant force (25 pN) (2). In previous studies, engineered coiled-coils were also shown to unzip nearly reversibly, and to generate significant folding forces while rezipping (24,25).

In addition to forming simple coiled-coils, α-helical polypeptides also form more complex structures in which nearly identical repeats composed of two or more antiparallel helices stack closely to generate spiral 3D structures (10,11,13). Because folded lengths of repeat proteins are on the order of 1/10 of the length of their polypeptide chains, repeat proteins promise to provide extreme stretch ratios when mechanically unfolded. However, in contrast to globular and fibrous proteins, the mechanical properties of these polypeptides remain largely uncharacterized. We recently

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used single-molecule atomic force spectroscopy to probe the elasticity of the spiral stack of 24 ankyrin-B repeats. We found that upon application of a sufficient stretching force, ANK repeats unfold one by one (26,27). Most remarkably, they refold rapidly and, in contrast to globular proteins, generate large refolding forces (26). Similar properties were recently observed for a small ankyrin repeat protein, gankyrin (28). Such unprecedented refolding properties promise to enable extreme stretch ratios with minimal energy dissipation, which may be advantageous for many mechanical functions, including mechanotransduction.

In this report, we investigate, at the single-molecule level, stretch ratios and energy dissipation during mechanical unfolding and refolding of a large class of repeat proteins composed of ANK and other repeats such as armadillo (ARM); Huntington, elongation factor 3, PR65/A subunit of protein phosphatase 2A, TOR (HEAT); and leucine-rich repeats (LRRs). Specifically, using single-molecule force spectroscopy, we executed stretch and relax measurements on 12 C-terminal (R13-24) ANK repeats of ankyrin-R (29,30), ARM repeats of β-catenin (31), HEAT repeats of clathrin (32), and LRRs of ribonuclease inhibitor (RI) (33) (Fig. 1; see Table S1 for more information on these proteins). ANK and HEAT repeats primarily comprise two antiparallel α-helices, ARM repeats primarily comprise three antiparallel α-helices, and LRRs of RI contain alternating α-helices and β-strands. Based on their crystal structures (30,32–34), and assuming that a single amino acid contributes 0.365 nm to the length of a stretched polypeptide chain (35), we anticipated that upon mechanical unfolding and stretching, these repeats could potentially support stretch ratios in the range of ~10–15.

MATERIALS AND METHODS

Cloning, expression, and purification of repeat proteins

Human β-catenin (residues 1–781), human clathrin (residues 1074–1522), and human RI (residues 1–461) were cloned into the pGex6p1-7his plasmid. The Ankyrin-R D34 gene (residues 402–827) was cloned into the pET-28a plasmid. Plasmids were transformed into *Escherichia coli* BL21-D3-pLysS. β-Catenin, clathrin, and RI were expressed for 3 h using isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. After harvesting and a freeze/thaw were completed, the lysates were spun down at 100 k xG for 1 h. For β-catenin and clathrin, the supernatants were first bound to nickel sepharose (catalog no. 17-5268-02; GE Healthcare Biosciences, Pittsburgh, PA), then washed, and eluted using imidazole. The eluted fractions were pooled and bound to glutathione Sepharose (catalog no. 17-5132-01; GE Healthcare) for 3 h and then washed extensively; proteins were cleaved from the glutathione S-transferase (GST) fusion using PreScission protease (catalog no. 270843; GE Healthcare). The GST-free protein was then chromatographed using a Superose 12 gel filtration column and an AKTA FPLC machine. Ankyrin-R D34 was transformed into *E. coli* BL21 and the cells were grown at 28°C for 4 h after induction with IPTG. His_6-tagged D34 was purified using a nickel affinity column (catalog no. 17-5248-01; GE Healthcare) followed by size exclusion chromatography (Superdex S75). Proteins were determined to be >95% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* expressing the RI was lysed in similar buffer with the addition of 0.5% sarkosyl and 10 mM dithiothreitol (DTT). After the 100 k xG spin, the lysate was coupled to the glutathione sepharose. The GST fusion was cleaved with PreScission protease, and the RI was adsorbed to an RNAase A affinity column, washed, and eluted using 4 M MgCl. The MgCl was dialyzed away before atomic force microscopy (AFM) measurements were performed. The RI was determined to be >95% pure by SDS-PAGE.

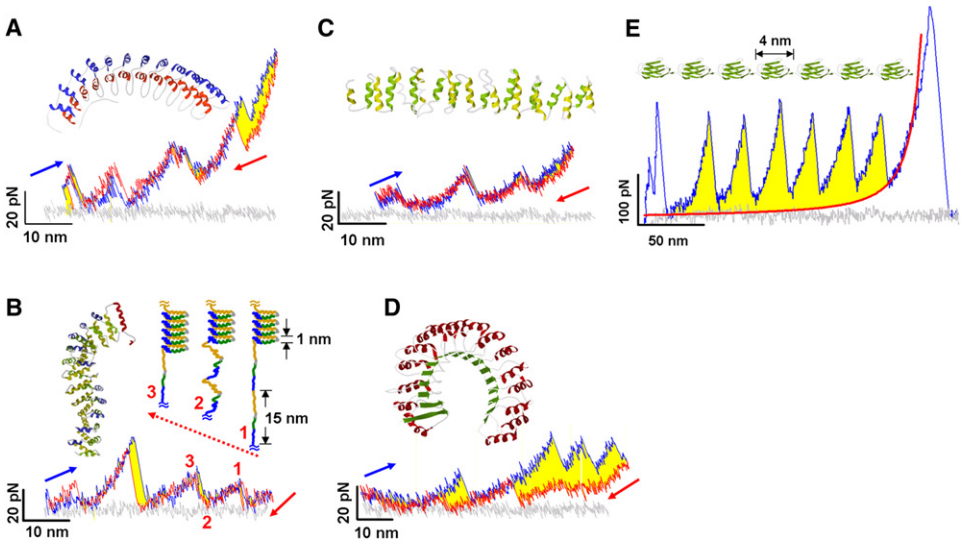

FIGURE 1 α-Helical repeat proteins do not dissipate much energy upon stretching and relaxing. (*A*) AFM-measured force-extension relationships of 12 ANK repeats of ankyrin-R (33 amino acids (aa) each), (*B*) 12 ARM repeats of β-catenin (~44 aa each), (*C*) 10 HEAT repeats of clathrin (~29 aa each), (*D*) 16 LR repeats of RI (~28 aa each), and (*E*) seven tandem repeats of the single Ig 27 domain $(I27)_7$. The stretching traces are marked in blue, relaxing traces in red, and the force baseline in gray. Extension rates ranged from 0.02 ~0.03 nm/ms for repeat proteins and ~0.2 nm/ms for $(I27)_7$. To calculate the amount of energy dissipated as heat, force-extension curves between stretching and relaxing traces are integrated (*yellow area*). When the systems are stretched and then released to their initial states, 7% (*A*), 10% (*B*), 3% (*C*), 50% (*D*), and 86% (*E*) of energy is dissipated. The insets show the crystal structures of the repeat proteins, D34 fragment of ankyrin-R (1N11), β-catenin (2Z6H), clathrin heavy chain (1B89), and RI (2Q4G), and $(I27)_7$. The inset in *B* represents a schematic of refolding of a single ARM repeat during β-catenin relaxation from stretching (15 nm → 1 nm). The refolding events marked 1, 2, and 3 correspond to the features of the refolding trace: 1→2 relaxing phase, 2→3 repeat refolding causes the AFM cantilever to snap back. (*E*) When $(I27)_7$ is stretched, six regular force peaks in a sawtooth pattern are obtained. Since this protein follows a simple polymer chain while relaxing (7,51), a WLC (*red curve*) was fit onto the curve of the fully stretched protein before it detached from the substrate or the AFM cantilever. When one I27 domain is mechanically unfolded and relaxed, ~350 kcal/mol is dissipated. More details pertaining to the methodology of single-molecule force spectroscopy can be found in the Supporting Material.

Sample preparation for AFM experiments

Stock protein solutions composed of 0.1–0.4 mg/mL of C-terminal polyhistidine-tagged ankyrin, clathrin, β-catenin, or RI were in solution containing 20 mM phosphate buffer, 200 mM NaCl, 1 mM EDTA, and 1–2 mM DTT (or 10 mM DTT for RI). For AFM stretching experiments, the solution was diluted to 1–10 μg/mL in phosphate-buffered saline (PBS) with 2–5 mM tris(2-carboxyethyl)phosphine (product No. 77720; Thermo Scientific, Rockford, IL) to prevent formation of disulfide bonds between molecules, and then deposited on various substrates for 30 min at room temperature. After incubation, the sample was washed three to four times with the buffer and then used for pulling experiments.

Immobilization of repeat proteins for force spectroscopy

To eliminate the possibility that the AFM-measured unfolding and refolding forces could be affected by the interaction between the protein and the AFM tip, or the protein and substrate to which it was attached, we used several different substrates (clean glass, clean gold, functionalized glass, and functionalized gold) and specific attachment of proteins to the tip as described below. With the exception of the initial pulls, which were affected by nonspecific adsorption of the tip and/or protein to substrate, as shown in Fig. S2 *A*, subsequent pulls in cyclic measurements produced similar force-extension curves regardless of the substrates and attachment methods used. Therefore, we concluded that the cyclic stretch-relax measurements that were performed on molecules lifted from the substrate were not affected by the type of substrate or the attachment method used.

To facilitate measurements on full-length proteins instead of their fragments, we also attempted to specifically attach one end to an Ni-NTA functionalized cantilever and the other end to a PEG-NHS substrate as described below. However, using this procedure, we still obtained recordings on fragments shorter than the full-length. This is possibly due to attachment of proteins to the PEG-NHS substrate through nonterminal amine groups.

Immobilization of repeat proteins on substrates

To prepare Ni-NTA substrates, glass cover slips were functionalized with the metal chelate N-nitrilotriacetic acid (NTA) as described previously (36). After chelating of Ni^{2+} on the NTA surface, the coverslips were stored in a desiccator at room temperature until use. The diluted protein solution was specifically bound to the Ni-NTA substrates by its C-terminal polyhistidine-tag during incubation for 30 min at room temperature. To prepare polyethylene glycol-*N*-hydroxysuccinimide (PEG-NHS) substrates, gold-coated glass coverslips were first sonicated in acetone, and then surface functionalization was performed as described previously (37). After COOH-$(PEG)_{24}$-NH_2 (product No. 26126; Thermo Scientific) was tethered onto 16-mercaptohexadecanoic acid (No. 448303; Sigma-Aldrich, St. Louis, MO), the substrates were stored in a desiccator at room temperature until they were used. To create stable amide bonds between the functionalized substrate and the amine groups of the proteins, the carboxyl ends of COOH-$(PEG)_{24}$-NH_2 were activated by 5 mM of sulfo-*N*-hydroxysulfosuccinimide (NHS; product No. 24510; Thermo Scientific) and 50 mM of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (No. E1769; Sigma) in 2-[morpholino] ethanesulfonic acid supplemented with 0.9% NaCl for 30 min. To prevent protein-protein connection by excess sulfo-NHS and EDC, the substrate was rinsed with PBS two to three times. Then, 50 μL of protein solution were deposited onto the substrate and incubated at room temperature for 30 min to create amide coupling. After incubation, the sample was gently washed three to four times with PBS buffer containing 2–5 mM tris(2-carboxyethyl)phosphine before it was used for the AFM pulling experiment.

Specific protein attachment onto cantilevers and PEG-NHS substrate

To prepare the Ni-NTA-functionalized cantilevers (OBL Biolevers; Veeco, Santa Barbara, CA) were cleaned with acetone and then incubated in HS-$(CH_2)_{11}$-EG_3-NTA (catalog No. TH016-002; Prochimia, Sopot, Poland) solution for 12 h to grow self-assembled monolayers onto the gold tips by thiol chemistry (38). To remove possible multilayers, the cantilevers were gently washed with ethanol. The cantilevers were submerged into a 10 mM $NiCl_2$ solution for 10 min and then used to produce specific binding between the Ni-NTA of the tip and polyhistidine-tag of proteins on the PEG-NHS substrate during AFM stretching measurements.

AFM stretching measurements

All stretching measurements were carried out on custom-built AFM instruments (20,39,40) equipped with an AFM detector head from Veeco Metrology Group, and high-resolution piezoelectric stages from Physik Instrumente (Karlsruhe/Palmback, Germany) equipped with capacitive or strain-gauge position sensors (vertical resolution of 0.1 nm). The spring constant of each cantilever Biolevers and Microlyers (MLCT-AUHW, Veeco) was calibrated in solution using the energy equipartition theorem as described previously (41). Proteins were picked up for stretching measurements by gently touching the substrate with the AFM cantilever tip, exploiting either nonspecific adsorption of the tip to the protein or specific binding of the Ni-NTA-functionalized tip to the polyhistidine-tag terminated proteins. Force-extension measurements were performed with AFM cantilevers at pulling speeds between 0.01 and 3 nm/ms, in solution and at room temperature, with a force resolution of ~5 pN and ~15 pN, respectively.

RESULTS AND DISCUSSION

α-Helical repeat proteins provide extreme stretch ratios with minimal energy dissipation

We begin by analyzing the mechanical unfolding and refolding properties of 12 ANK repeats of ankyrin-R, which is structurally similar to ankyrin-B (Fig. 1 *A*). We frequently observed stepwise unfolding of three to five ANK repeats of ankyrin-R. Upon relaxation of the stretching force, we observed rapid stepwise refolding of these repeats generating a force of 20–70 pN, similar to the unfolding force, which recapitulated the initial unfolding profile with minimal hysteresis (Fig. 1 *A*). In the stretch-relax cycle shown in Fig. 1 *A*, four ANK repeats were unfolded and stretched to ~10 times their folded length in a process that required an energy input of 228 kcal/mol (as determined from the area under the force-extension curve). Upon refolding, 212 kcal/mol was returned to the system. Thus, in this cycle, only 7% of the energy is dissipated as heat (the *yellow area* in Fig. 1 *A*). The finding that the unfolding/refolding results obtained for ankyrin-R repeats are similar to those obtained for ankyrin-B repeats (26) indicates that the two proteins possess similar mechanical properties, and implies that such properties may be characteristic of many ankyrin repeat domain proteins.

We then tested whether other ubiquitous repeat proteins with different folds possess similar nanomechanical properties. We found that β-catenin and clathrin also display stepwise unfolding upon stretching, and refold their repeats rapidly upon partial relaxation (estimated $t_{folding} < 5$ ms/repeat; Figs. 2, *A–C*, and 3, *A–C*; see Supporting Material). Refolding occurs against a significant load in a very similar pattern to the initial unfolding (Fig. 1, *B* and *C*), suggesting

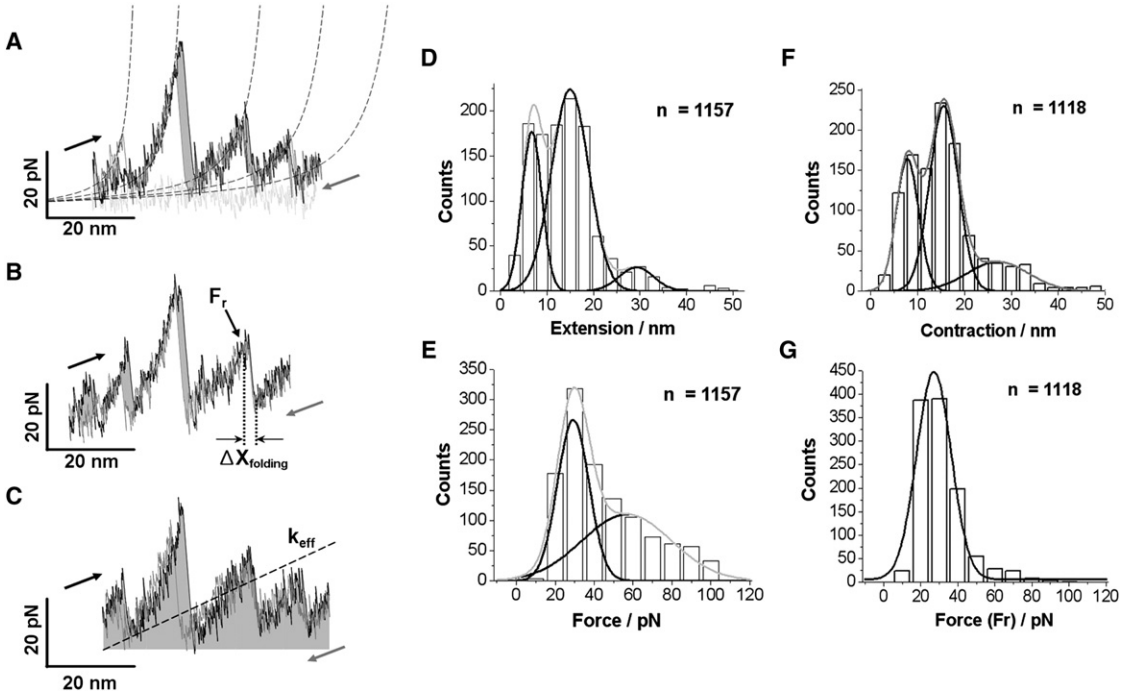

FIGURE 2 Fatigue endurance testing of β-catenin by cyclic stretch-relax experiments. (*A–C*) Only 8–15% energy is dissipated during cyclic measurements of the same molecule. The extension rate was 0.03 nm/ms. The stretching traces are marked in black, relaxing traces in dark gray, and the force baseline in light gray for Fig. 2, and Fig. 3. (*A*) The dashed black lines are WLC fits to the data with average contour length increment $\Delta L_c = 15.3 \pm 2.0$ nm, and persistence length p = 0.9 nm. The expected ΔL_c of ARM repeats in β-catenin is ~15 nm. This value corresponds to the average number of residues per repeat (44 aa × 0.365 nm/aa). (*B*) Folding lengths ($\Delta X_{folding}$) are measured to calculate refolding time (see Supporting Material), and maximum refolding forces (F_r) are measured from the zero-force baseline. (*C*) During stretching of the molecule, the potential energy (W) is calculated by integrating the force-extension unfolding curve (*gray area*; $W = \int F \times dx$; where F is the applied force and x is the extension), and then the effective spring constant (k_{eff}, *dashed black line*) is calculated by $k_{eff} = 2 \times W/x^2$. (*D–G*) We analyzed similar behaviors of unfolding/refolding forces and extension/contraction lengths in independent measurements on 51 different molecules. Unfolding contour length increments (*D*) and refolding contour length decrements (*F*), measured by the WLC model, were fitted with multiple Gaussian distributions. The average fitted lengths are 6.7 ± 0.2, 15.0 ± 0.2, and 29.2 ± 1.4 nm in panel *D*, and 7.9 ± 0.1, 15.6 ± 0.1 and 27.1 ± 1.0 nm in panel *F*, which are comparable to the length of a helix, an individual repeat, and two repeats, respectively. In *E*, two Gaussian distributions fitted with the average unfolding forces of 29.2 ± 0.7, and 56.8 ± 6.4 pN, whereas in *G*, a single Gaussian distribution fitted with the average refolding force of 27.1 ± 0.7 pN.

that the mechanical unfolding occurs as a quasi-equilibrium process with minimal energy dissipation. Similarly reversible behavior was previously observed for myosin undergoing stretch/relax cycles (2) and synthetic coiled-coils undergoing unzipping/rezipping cycles (24,25).

α/β Polypeptides reveal significant mechanical hysteresis

The LR domain of RI does not refold as robustly after relaxation of the stretching forces (Fig. 1 *D*, Fig. S4). In the case shown in Fig. 1 *D*, 50% of its stretch energy was dissipated as heat. The lack of reversibility in the unfolding of LR repeats may reflect the generally slower folding rates observed with β-structures relative to α-helices (7,42,43). It is interesting that other, purely α-helical repeat proteins that do not form spiral tertiary structures, such as spectrins, also display less robust mechanical refolding (44–46). This observation suggests that the arrangement of repeats into a tightly packed stack is a preferred structural element for equilibrium unfolding and for refolding force generation.

α-Helical repeat proteins display little mechanical fatigue

To confirm our results, we analyzed hundreds of stretching and relaxing recordings similar to those shown in Fig. 1. We show the distributions of unfolding and refolding forces measured from the zero-force baseline, and of extension and contraction lengths measured by worm-like chain (WLC) fits to the force peaks in Figs. 2, *D–G*, and 3, *E–H*. The histograms of unfolding/refolding lengths shown in Figs. 2, *D* and *F*, and 3, *E* and *G*, suggest that the vast majority of these mechanical events involved whole repeats or their α-helical subunits. The histograms of unfolding forces in Figs. 2 *E* and 3 *F* reveal a major peak at ~30 pN and a secondary peak at ~60 pN, suggesting that the former corresponds to the unfolding of one repeat and the latter likely represents the force necessary to simultaneously unfold two repeats. Also, based on the amount of work necessary to extend these polypeptides, we determined their effective spring constants, k_{eff}, to be in the range of 0.5–2 pN/nm (Fig. 2 *C*).

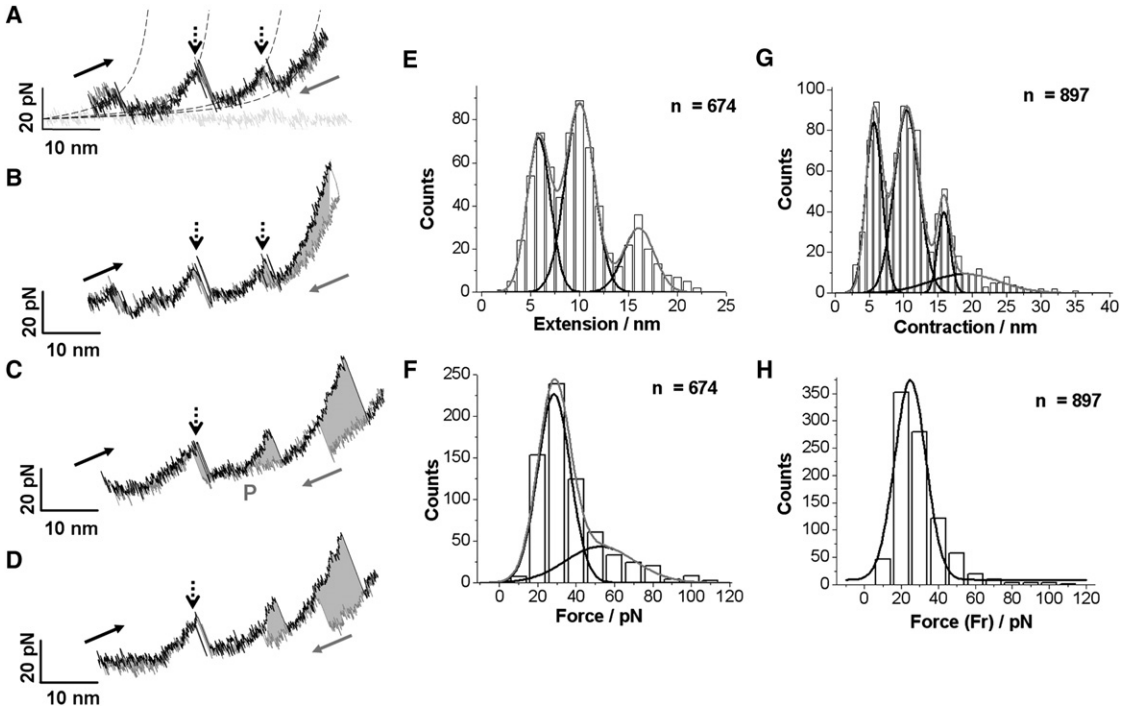

FIGURE 3 Cyclic stretch-relax experiment on clathrin heavy chain repeats. (*A–D*) During cyclic measurements on the same molecule, 0–25% of the energy was dissipated. The extension rate was 0.03 nm/ms. (*A*) The dashed black lines are WLC fits to the data with $\Delta L_c = 11.3 \pm 2.1$ nm, and $p = 1$ nm. (*B* and *C*) Hysteresis becomes pronounced in *B* due to an increase in the extension relative to *A*. The plateau force feature, P, is captured while relaxing in *C*, and the force peak is recovered in *D*. (*E* and *F*) We observed similar behavior in independent measurements on 63 different clathrin molecules. Unfolding contour length increments (*E*) and refolding contour length decrements (*G*), measured by the WLC model, are fitted with multiple Gaussian distributions. The average fitted lengths are 5.8 ± 0.1, 10.0 ± 0.1, and 16.1 ± 0.1 nm in *E*, and 5.7 ± 0.1, 10.1 ± 0.1, 15.9 ± 0.1, and 19.3 ± 4.3 nm in *G*, which are comparable to the length of a helix, an individual repeat, three helixes, and (during refolding) two repeats, respectively. In *F*, two Gaussian distributions fitted with the average unfolding forces of 28.5 ± 0.5, and 52.3 ± 13.0 pN, whereas in *H*, a single Gaussian distribution fitted with the average refolding force of 24.8 ± 0.7 pN.

We then cyclically stretched molecules to test their fatigue resistance. Figs. 2, *A–C*, and 3, *A–D*, show the results of elasticity measurements of ARM and HEAT repeats obtained through repetitive stretching and relaxation cycles performed on the same molecule. Generally, the force-extension curves obtained at the beginning of the cycle are similar to those obtained after the molecule was stretched and relaxed multiple times. This result suggests high endurance properties, with stretch ratios approaching 1500% and 1000% for ARM and HEAT repeats, respectively.

However, we occasionally observed some hysteresis between stretching and relaxing the molecule, the magnitude of which seemed to increase with the increased number of repeats being unfolded in the stretching part of the cycle (Fig. 3, *B–D*, and Fig. S2 *B*). These initial observations suggest that the speed and fidelity of refolding may be affected by the number of repeats that remain folded at all times and thus serve as a folding template. This conjecture has potential implications for the biological functions and evolution of the numerous natural repeat proteins, and will be systematically tested in future studies.

After the onset of hysteresis, the refolding traces occasionally missed some of the force peaks captured during the stretching part of the cycle, and in their place there was a force plateau (the feature marked as P in Fig. 3 *C*). It is interesting that such a plateau was captured intermittently in consecutive stretch-relax measurements even on the same molecule and was later replaced by a regular refolding force peak (Fig. 3 *D*). In single-molecule force spectroscopy of biopolymers, such as DNA (47,48), polysaccharides (49), and coiled-coils (2), a force plateau may be associated with the recoiling of the polymer, when helical structures are formed, because this process seems to maintain an almost constant tension in the polymer while its length contracts (2,47,49). Thus, we hypothesize that the force plateau in the relaxing force curves of clathrin captured the formation of its α-helical units before they later collapsed into the final HEAT repeat structure.

Fine details of unfolding and refolding events are captured by AFM

The unfolding and refolding traces of the repeat proteins captured by AFM generally display a set of sharp force peaks (Fig. 1, *A–C*), suggesting that the unfolding/refolding of repeats may occur in a single step. However, some of the captured unfolding and refolding force peaks were broad, and upon closer inspection revealed a fine substructure. These

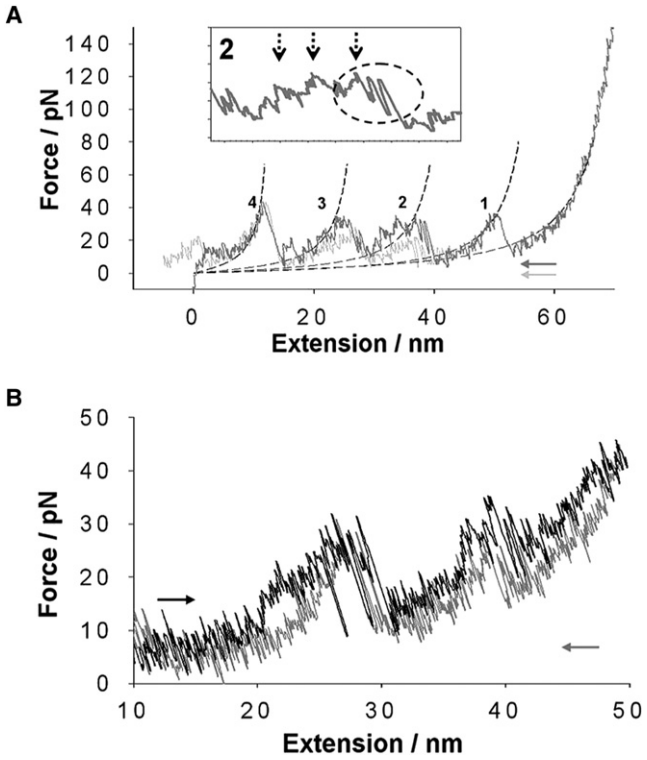

FIGURE 4 Fine details of unfolding/refolding force peaks of β-catenin (*A*) and clathrin (*B*) indicate partial repeat unfolding/refolding and fluctuation between folded and unfolded states. The stretching traces are marked in black, and relaxing traces are plotted in dark gray and gray. (*A*) Comparison of two refolding traces obtained on two different molecules with sharp force peaks (*gray trace*, same as the *red trace* in Fig. 1 *B*) and broad force peaks *(dark gray trace* and *inset*). The extension rate for the dark gray curve was 0.02 nm/ms. The dashed black lines are WLC fits to the refolding data shown in dark gray, with $\Delta Lc = 15.5 \pm 1.2$ nm and $p = 1$ nm. The inset shows refolding peak 2 at a higher magnification, revealing three smaller peaks indicated by the dotted black arrows. The black dashed circle marks transient refolding and unfolding events of an ARM repeat. (*B*) Similar transient unfolding and refolding events are also captured for clathrin during its stretching and relaxing (see also *dotted black arrows* in Fig. 3, *A–D*). The extension rate for this measurement was 0.03 nm/ms.

broad features were actually composed of doublets or triplets of smaller peaks (e.g., refolding peak 2 in Fig. 4 *A* and in the *inset*). An analysis of the spacing among these small peaks suggests that they may represent a consecutive refolding of three helices within one ARM repeat, and hence that AFM is able to resolve refolding intermediates of these repeats.

We also note that some unfolding and refolding traces of ARM and HEAT repeats reveal unusual force and length oscillations (for example, see the feature within the *dashed circle* in the *inset* of Fig. 4 *A*; Fig. 4 *B*, and *black dotted arrows*) in Fig. 3, *A–D*), suggesting that transient unfolding/refolding events took place. A similar behavior was recently observed during mechanical unfolding of individual calmodulin molecules (50). It seems that by mechanical unfolding and refolding of whole repeats or individual α-helices, repeat proteins can quickly fluctuate between states with varying end-to-end lengths and tensions. Such an oscillatory behavior that can exert tight control over the molecule's length and tension while conserving the mechanical energy may be advantageous for mechanotransduction. Further work will be necessary to identify unfolding and refolding intermediates, and to understand the mechanism of transient events during cycling stretching of these repeats and their assemblies.

Possible use of repeat proteins as bio-inspired springy materials and force sensors

Tunable properties such as length, stiffness, and extensibility could be designed with the use of engineered linear or torsion spring structures composed of ANK, ARM, or HEAT repeats. Monomeric stacks composed of various numbers of these repeats could be interconnected using thiol-disulfide, olefin metathesis chemistry, or by other side-chain-enabled cross-linking, into various nanostructures. Such structures are anticipated to display extreme stretch ratios and reversibility characteristic of their monomeric components, making these structures particularly useful for applications involving high strains and cyclic loading. The incorporation of reporter functionalities (e.g., fluorescence resonance energy transfer pairs at each end of three to four repeats) could provide a basis for using these synthetic proteins as time-resolved force nanosensors.

CONCLUSIONS

Based on the results presented here, we propose that α-helical repeats that form spiral structures constitute a novel, hitherto unrecognized (to our knowledge) class of polypeptides with extremely useful properties, such as high stretch ratios with minimal energy dissipation. Such properties may be ideal for supporting various functions of these proteins, including mechanotransduction, and may be also exploited for novel bio-inspired materials. The physical model of protein elements of cellular structures under tension needs revision to incorporate spring behavior.

SUPPORTING MATERIAL

Four figures and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00348-6.

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