Prying Open Single GroES Ring Complexes by Force Reveals Cooperativity across Domains

Akiko Ikeda-Kobayashi,† Yukinori Taniguchi,† David J. Brockwell,§ Emanuele Paci,§ and Masaru Kawakami*†
†School of Materials Science, Japan Advanced Institute of Science and Technology (JAIST), Ishikawa, Japan; †Japan Society for the Promotion of Science (JSPS), Tokyo, Japan; and §Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom

ABSTRACT Understanding how the mechanical properties of a protein complex emerge from the interplay of intra- and interchain interactions is vital at both fundamental and applied levels. To investigate whether interdomain cooperativity affects protein mechanical strength, we employed single-molecule force spectroscopy to probe the mechanical stability of GroES, a homoheptamer with a domelike quaternary structure stabilized by intersubunit interactions between the first and last β-strands of adjacent domains. A GroES variant was constructed in which each subunit of the GroES heptamer is covalently linked to adjacent subunits by tripeptide linkers and folded domains of protein L are introduced to the heptamer’s termini as handle molecules. The force-distance profiles for GroES unfolding showed, for the first time that we know of, a mechanical phenotype whereby seven distinct force peaks, with alternating behavior of unfolding force and contour length (ΔLc), were observed with increasing unfolding-event number. Unfolding of (GroES)7 is initiated by breakage of the interface between domains 1 and 7 at low force, which imparts a polarity to (GroES)7 that results in two distinct mechanical phenotypes of these otherwise identical protein domains. Unfolding then proceeds by peeling domains off the domelike native structure by sequential repetition of the denaturation of mechanically weak (unfoldon 1) and strong (unfoldon 2) units. These results indicate that domain-domain interactions help to determine the overall mechanical strength and unfolding pathway of the oligomeric structure. These data reveal an unexpected richness in the mechanical behavior of this homopolyprotein, yielding a complex with greater mechanical strength and properties distinct from those that would be apparent for GroES domains in isolation.

INTRODUCTION Proteins are fundamental components of life with functions that span catalytic, signaling, and structural roles. Over the last decade it has become clear that mechanical deformation can be used to modulate these properties in vivo (1–5). Understanding the determinants of mechanical stability and the effects of force on the dynamic properties of protein molecules are thus important both at a fundamental level and in the design of artificial biomaterials with tuned mechanical and dynamic properties (6). To date, most single-molecule mechanical unfolding studies have focused on single- or multidomain monomeric proteins, as these are easy to study, especially when concatenated into a tandem array (7). Most proteins, however, do not act as isolated monomers, but associate and work in an oligomeric form. An oligomer is usually formed by noncovalent protein-protein interactions between subunits with high specificity and affinity that provides a characteristic quaternary structure and complex function. In contrast to the polypeptides typically used in single-molecule atomic force microscope (AFM) unfolding experiments, where interdomain interactions are purposely minimized, most oligomers form complex structures that sequester large areas of protein surface away from solvent. How complex formation affects the mechanical properties of proteins is unknown, but it is necessary to measure these effects to understand how the macroscopic properties of a complex emerge from the convolution of the intra- and intermolecular interactions.

The homoheptamer GroES is a cochaperonine protein that forms a complex with GroEL, which regulates the folding of nascent polypeptides into natively folded proteins. The crystal structure of GroES (8) shows a homoheptameric protein composed of 10-kDa subunits, forming a domelike ring structure stabilized by hydrogen bonds and hydrophobic interactions between the first β-strand of one subunit and the last β-strand of an adjacent subunit. The thermodynamic stability of the equilibrium between the unfolded/folded monomer and the folded heptamer of GroES has been studied by various techniques, such as chemical denaturation (9–11), calorimetry (12), dilution (13,14), and hydrostatic pressure (15). We anticipate that these interactions also contribute to the mechanical stability of the heptameric GroES ring, a question that single-molecule force spectroscopy is able to address.

Sakane et al. (16) previously used AFM to investigate the mechanical stability of a single-chain variant of heptameric GroES (denoted here as sc(GroES)7) after immobilization on a mica substrate. Very few unfolding events were recorded, however, and the results were inconclusive. Here, we increase the rate of successful pulling of a GroES heptamer and identify true sc(GroES)7 unfolding events by
introducing protein L domains as handle molecules at the N- and C-termini of sc(GroES)7. This approach reveals what to our knowledge is a novel force response for a protein whereby two distinct mechanical phenotypes are observed for GroES domains as the quaternary structure is unraveled.

MATERIALS AND METHODS

Cloning and expression of a GroES-protein L heteropolyprotein

A heptamer of GroES domains was expressed as a heteropolyprotein alongside protein L, a protein of known mechanical properties (17,18). The expression plasmid was constructed using a modular cassette approach using p(protein L)5 as a scaffold (17). This expression vector encodes five protein L domains, an N-terminal hexahistidine tag for purification, and two C-terminal cysteine residues for immobilization. A heptamer of GroES domains linked by their C- and N-termini by a three glycine linker was constructed as described previously (16) and inserted into position 3 of p(protein L)2 to yield p(protein L)2(GroES)-protein L)2. The amino acid sequence of the linkers between each cassette is MHHHHH HSSGG-(protein L)-GLVEARGG-(protein L)-GLIERARGG-(protein L)-GLISARHMPGGG-(GroES)-amino acid sequence of the linkers between each cassette is MHHHHH HSSGG-(protein L)-GLVEARGG-(protein L)-GLIERARGG-(protein L)-GLISARHMPGGG-(GroES). Biophysical Journal 102(8) 1961–1968

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Mechanical unfolding experiments were performed using a molecular force probe 1D (Asylum Research, Santa Barbara, CA) mounted with gold-coated silicon nitride micro cantilevers (Biolever B, Olympus Optical, Tokyo, Japan) with spring constants of ~8 pN/nm (nominal spring constant value is 6 pN/nm). The spring constant of each cantilever was determined by the thermal noise method (19) before data accumulation. Heteropolypeptides were immobilized onto a gold derivitized surface via two C-terminal Cys residues by pipetting 100 μl of protein solution (0.1 mg/ml) onto the freshly template-stripped surface and then incubating for 20 min at room temperature. The gold surface was then rinsed with phosphate-buffered saline (Nacalai tesque) to remove nonspecifically bound proteins. Mechanical unfolding data were accumulated at a retraction speed of 230 nm/s.

Unfolding data analysis

Estimation of changes in contour length

Contour lengths (Lc) were calculated by fitting unfolding events to a wormlike chain model:

\[ F = \frac{k_BT}{\rho} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right)^2 - \frac{1}{4} + \frac{x}{L_c} \right], \]

where \( F \) is the force at extension \( x \), \( k_B \) is the Boltzmann constant, \( T \) is temperature, and \( p \) is the persistence length. In this study, \( p \) was fixed at 0.6 nm. The change in contour length upon unfolding (ΔLc) was calculated by subtracting the contour length obtained from a fit of one unfolding event from the contour length obtained from a fit of the next unfolding event.

Estimation of length increase (ΔLc) upon unfolding of GroES and protein L

The values of contour-length increase (ΔLc) corresponding to unfolding events of individually separated subunits of protein L and GroES were

![Figure 1](https://example.com/figure1.png)

(a) Design of (protein L)2(GroES)7-(protein L)2 at the DNA level. Unique pairs of restriction endonuclease sites (arrows) define each cassette of protein L monomer (hexagons) or heptamer of GroES subunits (rectangles). The hexahistidine tag at the N-terminus permits facile purification, and two C-terminal cysteine residues allow covalent immobilization of the protein onto a gold substrate. (b) SDS-PAGE gel of (protein-L)2(GroES)7-(protein L)2 purified by Ni-NTA and size-exclusion chromatography. The predicted molecular mass of the chimera protein is 111 kDa. The righthand lane is a broad-range molecular mass marker (New England Biolabs, Ipswich, MA). (c) Schematic illustration of the experimental setup. The three-dimensional structure of protein L (gray) and the GroES heptamer (subunits identified by different-colored spheres) are drawn with Molfeat (flat lucx, Tokyo, Japan) using coordinates from PDB files 1HZ6 and 1AON. (d) Superposition of typical force-extension traces with unfolding events for each of the seven GroES subunits (0–175 nm) followed by four protein L unfolding peaks.

Continuous gray and dotted lines are WLC fits. For reference, vertical lines are drawn at 30 nm and 60 nm from the surface (see text).
estimated to be 16.9 and 29.7 nm, respectively. These values were obtained by subtracting the through-space distance between the N- and C-terminal residues of the structured core of each protein (3.5 and 3.3 nm for protein L and GroES, respectively) from the length of the fully extended state of each protein (0.34 nm/residue multiplied by the numbers of amino acids within the folded domain, which are 60 and 97 for protein L and GroES, respectively).

The above method assumes that each monomer is arranged in a linear bead-on-a-string manner. However, sc(GroES)7 is thought to form a ringlike structure identical to that observed for the wild-type heptamer (16) that is stabilized by intersubunit interactions between the N- and C-terminal strands of adjacent subunits. In this case, estimation of the contour length increase of GroES oligomers (Fig. 2) was calculated using cleft angles of 0°, 10°, 20°, and 30° (see Fig. S1). Briefly, in these calculations, the molecular length of the GroES ring structure is considered as a heptagonal pyramid with the length of a side of the base equal to 3.3 nm (Fig S1 b, right), and the end-to-end distance of oligomers with a cleft was estimated by a simple geometrical calculation for the opening of the seven triangles in a plane, as shown in Fig. S1, c and d. Theta is the cleft angle, n is the number of folded subunits, and x is the base angle of the triangle. The angles a and b are obtained by determining the cleft angle, θ, using the equations

\[
x = \frac{180 - \frac{360}{7}}{2},
\]

\[
a = 180 - \theta - 2x,
\]

\[
b = 90 - \frac{\theta}{2} - x,
\]

\[
L_n = 3.3 + 2 \times 3.3 \times \sum_{i=1}^{(n-1)/2} \cos(i \times a) \quad (n = 1, 3, 5, 7),
\]

\[
L_n = 2 \times 3.3 \times \sum_{i=1}^{n/2} \cos[(i-1)a + b] \quad (n = 2, 4, 6).
\]

Then, the molecular length difference between an nmer and an (n – 1)mer is calculated as

\[
\Delta L_n = L_n + (0.34 \times 3) - L_{n-1},
\]

where the length of a linker of three glycine residues between subunits is taken into account, using a value of 0.34 nm for the length of a single peptide unit.

**Determination of the modal unfolding force**

Unfolding events were identified as those for protein L or GroES based on the ΔLn value for each event. Unfolding force-frequency histograms were then constructed as a function of the event number (for GroES unfolding) or pooled for all event numbers (protein L). The modal unfolding force

**FIGURE 2** (a and b) Unfolding force (a) and ΔLn (b) distributions of single GroES domains, drawn with respect to the order of unfolding events (top to bottom, peaks 1–7). Fits to the data using Eq. 8 (a) and a Gaussian function (b) are shown as solid lines. (c and d) ΔLn (c) and unfolding force (d) distributions of protein L domains. Solid lines show a fit with Gaussian function (c) and Monte Carlo simulation results ((d); see text). (e and f) Modal unfolding forces (e) and ΔLn (f) of GroES domains plotted as a function of unfolding event. Insets show modal unfolding force (e, open circles) and ΔLn (f, solid diamonds) of protein L as a function of unfolding event. The calculated ΔLn, with the assumption that the ring structure of the GroES oligomer is moderately opened, with a cleft angle of 0° (complete ring), 10°, 20°, and 30° (see Supporting Material) are shown as open circles, triangles, squares, and cross markers, respectively. Asterisks indicate the calculated ΔLn for GroES domains arranged in a bead-on-a-string manner. Dashed line in f indicates the ΔLn for GroES domains assuming that the sc(GroES)7 ring has a cleft angle of 20° and unfolds by alternation of unfoldon 1 and unfoldon 2 events (see text).
for each force-frequency histogram was then calculated by fitting each distribution with the equation (20)

\[
\frac{dF_u}{dF} = \frac{k_u}{V_f} \exp\left(\frac{F_{x_u}}{k_uT}\right) \exp\left(\left(\frac{k_u}{V_f x_u}\right) \left(\exp\left(\frac{F_{x_u}}{k_uT}\right) - 1 \right)\right).
\]

This equation is derived assuming the probability density for the unfolding of a folded domain, \( P_u \), at constant force loading rate (force ramp, \( V_f \), \( x_u \), and \( k_u \) are the distance between the native state and the transition state, and the unfolding rate constant at zero force for the mechanical unfolding of a folded domain, respectively. \( V_f \), \( x_u \), and \( k_u \) are left unconstrained for fitting. This equation is used only for generating the skewed curve that fits well to the experimental distributions. The modal force is defined as the maximum of the fitted curve.

**Molecular dynamics simulations**

Molecular dynamics simulations were performed using the implicit solvent model FACTS (21) implemented in CHARMM (22). Implicit solvent models are computationally efficient and avoid artifacts due to the relaxation of the explicit solvent, which might be slow relative to the fast conformational changes induced by the external force. The starting configuration for the heptamer was generated using the structure of GroES/EL complex (PDB accession code 1AON (23)) in which each GroES domain is tandemly linked by three glycine residues, as in the experiment. The initial structures of unfoldons 1 and 2 were obtained by selecting residues 9–100 and 1–110, respectively, of the heptamer (using the numbering from PDB file 1AON). The N- and C termini of the first and seventh domains are pulled apart through a harmonic spring displaced at constant velocity. We used spring constants between 10 pN Å⁻¹ and constant velocities in the range 10⁻⁵–10⁰ nm s⁻¹. Unfolding forces, as in the experiment, correspond to the maximum force in the force-extension profile. Simulations were performed at 300 K with Langevin dynamics in low solvent-viscosity conditions.

**RESULTS AND DISCUSSION**

In protein mechanical unfolding experiments using an AFM, the tip can bind anywhere along the length of the protein that is immobilized on the substrate. Nonspecific protein-substrate interactions that commonly occur close to the surface can thus complicate the interpretation of force-extension profiles. To identify force-extension profiles that reported on the unfolding of all seven domains of GroES, a chimeric polyprotein, \((\text{protein L})_2(\text{GroES})_7(\text{protein L})_2\), was constructed (see Fig. 1, a–c) by insertion of a single-chain variant of the GroES heptamer (scGroES)₇ at position 3 of a previously reported protein L pentamer (17). Force-extension profiles containing four protein L unfolding events (assigned by the known \( \Delta L_c \) (17)) must therefore also report on the unfolding behavior of (GroES)₇. The single-chain (GroES)₇ used in this study is identical to that previously reported by the Kawata group, and this variant was found to be structurally and functionally indistinguishable from the wild-type heptamer (16).

After purification to homogeneity (Fig. 1b) single molecules of \((\text{protein L})_2(\text{GroES})_7(\text{protein L})_2\) immobilized onto a gold substrate were picked up with an AFM cantilever tip and stretched for several hundred nanometers at a constant speed of 230 nm/s. Typical force-extension profiles containing four protein L unfolding events are superimposed in Fig. 1d. Each profile exhibits a characteristic saw-toothed pattern with seven successive unfolding events at low force (modal force = 35.3 pN) and four evenly spaced unfolding events (\( \Delta L_c = 16.8 \pm 0.2 \) nm) at higher force (modal force = 94.6 pN) followed by a single protein-tip/substrate detachment event at high force. Values of \( \Delta L_c \) and unfolding force of the four similar high-force unfolding events observed here give similar values for \( \Delta L_c \) (18.0 ± 1.3 nm) but significantly lower unfolding forces compared to those previously published for protein L (125 pN). However, the modal unfolding force for a protein is dependent on the force loading rate and the composition of the construct (number and size of each monomer within the polyprotein). Modeling these effects by Monte Carlo simulations using previously obtained parameters for protein L (\( k_u = 0.05 \) s⁻¹ and \( x_u = 0.22 \) nm (17)) predicts a modal unfolding force of 103 pN, similar to the value reported here. These observations verify the assignment of high-force events to protein L unfolding. Consequently, given the design of \((\text{protein L})_2(\text{GroES})_7(\text{protein L})_2\), the first seven low-force unfolding peaks would be expected to be those for the unfolding of single GroES domains. Indeed, \( \Delta L_c \) for these events are significantly longer (~30 nm (Fig. 1d)), which roughly corresponds to the value expected for a 97-residue globular protein (29.7 nm, assuming a bead-on-a-string arrangement, see **Materials and Methods**). The relatively low mechanical strength of GroES subunits accords with its mixed secondary structure and the arrangement of these structural elements relative to the pulling direction, as observed previously for other proteins with mixed α/β and α + β structures, such as MBP (24,25), DHFR (26,27), and lysozyme (28,29). However, the unfolding values reported here are significantly smaller than the ~50 pN reported previously (30) for scGroES)₇. This probably reflects the fact that the different constructs used required different methods of surface attachment, leading to differences in the ability to identify bona fide unfolding events. In this study, it can be seen that nonspecific protein/tip/surface interactions are still evident close to the surface (Fig. 1d). However, for the construct used here, filtering the data and aligning force-extension profiles by using the mechanical fingerprint of protein L unfolding events reveals, remarkably, that the unfolding forces for identical GroES domains alternate between low (31.24 ± 0.65 pN, \( N = 241 \)) and high (40.33 ± 0.78 pN, \( N = 196 \)) values. Such behavior is absent for protein L unfolding events (see later).

To quantify this novel (to our knowledge) observation, the frequency distribution of the unfolding forces and \( \Delta L_c \) values for GroES subunits were analyzed as a function of event number (Fig. 2, a and b). Surprisingly, in addition to the alternation in unfolding forces (Fig. 2, a and e), which is evident in the force-extension profiles, \( \Delta L_c \) values also alternate between low (28.81 ± 0.47 nm, odd event number, \( N = 237 \)) and high (35.38 ± 0.41 nm, even event number, \( N = 183 \)) values so that low unfolding forces correlate
with shorter unfolding distances (Fig. 2, b, e, and f). In addition to alternation in the modal force and $\Delta L_{e}$, the widths of the frequency distributions are significantly wider for event numbers with high modal unfolding forces relative to those for low forces. These data suggest some cooperativity across pairs of GroES subunits giving two distinct mechanical phenotypes with differently sized mechanically resistant cores that unfold over distinct energy landscapes (both $k_u$ and $x_u$ are different). This contrasts with the mechanical behavior of tandemly arrayed monomeric proteins (such as the protein L domains in this study, Fig. 2, c and d, and Fig. 2, e and f, insets). The unfolding force for protein L shows a gradual increase in unfolding force, due to the number-of-folded-domains effect (31), whereas $\Delta L_{e}$ is independent of event number.

The cooperativity across subunit interfaces suggests that some (or all) of the seven domain/domain interfaces retain their contacts before unfolding. As described above, sc(GroES)$_7$ appears to be identical to (GroES)$_7$ both functionally and structurally (16) suggesting the formation of the native domelike structure (8,23). Here, sc(GroES)$_7$ is sandwiched between four protein L domains via 8 and 12 residue linkers. These spacers minimize aberrant protein interactions, evidenced by the similarity of protein L modal unfolding force (97 pN) to that determined by a Monte Carlo simulation (103 pN; Fig. 2 d, red line) using parameters published previously for protein L (see above). It is thus reasonable to assume that the native quaternary structure of GroES is formed. To verify this assumption, the $\Delta L_{e}$ between fully folded and fully extended, unfolded conformations of (protein L)$_2$(GroES)$_7$(protein L)$_2$ (i.e., the $\Delta L_{e}$ between event 1 and the tip/substrate-protein rupture event) was measured (300.3 $\pm$ 1.3 nm) and compared to that estimated for (protein L)$_2$(GroES)$_7$(protein L)$_2$ starting with sc(GroES)$_7$ in nativelike and bead-on-string arrangements (305.6 and 276.4 nm, respectively). The similarity of the measured $\Delta L_{e}$ to that estimated for the nativelike conformation strongly suggests that a nativelike structure is present at the onset of unfolding. To test this hypothesis further, a steered molecular dynamics simulation was performed with sc(GroES)$_7$, in which each domain is linked to the adjacent one by three glycine residues and the N- and C-termini of the first and seventh domains are pulled apart at constant velocity (Materials and Methods and Fig. 3 a). In the beginning of the stretch, we observe that the ring structure is opened without any domain unfolding. Instead, each domain interface is pried apart slightly, followed by sequential domain unfolding that proceeds from the terminal domains. If we assume that the ring structure is maintained and that upon extension the outermost subunit unfolds, at early unfolding events the contour length increase should give higher values than that predicted for the polypeptide chain length of a single GroES domain (29.7 nm). Consequently, the calculated $\Delta L_{e}$ decreases as the unfolding-event number increases. We have calculated the $\Delta L_{e}$ for a completely closed GroES ring as a function of unfolding-event number (Fig. 2 f, open circles). In addition, we have calculated $\Delta L_{e}$, assuming that the GroES ring has a soft structure, in which the folded domain retains its rigid structure but extension induces a cleft with an angle of $\theta$ between the subunits. The decrease in $\Delta L_{e}$ as a function of event number lessens as the cleft angle is increased (Fig. 2 f). The median of the alternating $\Delta L_{e}$ values is found between the models with $\theta = 10^\circ$ and $20^\circ$ (triangles and squares, respectively). Upon extension, the GroES ring is opened by a mechanical force. The first unfolding event has a modal unfolding force of 34 pN. At the moment of the first unfolding event, the ring structure is opened with a cleft angle of 15$^\circ$ between subunits, giving an open-jaw conformation with an extension of 8.4 nm (from Eq. 5). From these values, we can estimate the spring constant of the ring structure to be 34/8.4 = 4.0 pN nm$^{-1}$. This value is much smaller than the previously reported spring constant for globular folded proteins (estimated to be hundreds to thousands of pN nm$^{-1}$) (32) but close to that for proteins with springlike properties such as ankyrin (33). Although it is unlikely that the folded GroES subunits are soft, the interaction between the subunits may give pliability to the heptamer ring structure. The high pliability of the GroES ring structure has been proposed to be due to weak packing and electrostatic repulsion between the $\beta$-hairpins, which form the orifice of the GroES ring (8). Indeed, in some studies, the radius of gyration of GroES in solution determined by SAXS showed a higher value than that calculated from the crystal structure (16,34).
The ability to unequivocally identify sc(GroES)$_7$ unfolding events has revealed a disparity between the force and $\Delta L_c$ frequency distributions as a function of event number. Comparison of these parameters for GroES and protein L unfolding events (Fig. 2, e and f) clearly indicate novel mechanical behavior for GroES whereby the stepwise change in oligomeric state of GroES modulates the mechanical strength of the remaining nativelike domains. Native-like (GroES)$_7$ comprises seven identical domains, apparently contradicting the observation above. What is the cause of the differing mechanical behavior that is evident in the force-extension profiles? We have demonstrated above that sc(GroES)$_7$ within its protein L scaffold is in a nativelike configuration at the onset of force application. When extended in the context of (protein L)$_2$ (GroES)$_7$(protein L)$_2$, a peeling force is applied between the N-terminus of strand 1 of the first GroES domain and the C-terminus of strand 9 of the seventh GroES domain. This type of deformation can result in unfolding forces below the thermal noise limit of the technique (7,35) fracturing the ringlike structure (Fig. 3 a). It is important to note that the N- and C-terminal residues of adjacent GroES monomers form zippered antiparallel $\beta$-strands (8) (Fig. 3, b and c) that are known to play an important role in heptamer assembly and stability. For example, deletion of the seven C-terminal residues prevents (GroES)$_7$ assembly (36), whereas variants that contain hydrophobic deletions in the N- and C-terminal strands decrease the stability of the complex (37). Breaking the interface between domains 1 and 7 would thus impart a polarity to sc(GroES)$_7$, destabilizing both terminal subunits. Given that the stabilizing interfacial contacts occur throughout the unfolding pathway, it appears that the domelike structure of sc(GroES)$_7$ is denatured in a stepwise manner progressing from one of the destabilized terminal subunits. The discussion below assumes that the destabilized C-terminal GroES domain lacking the stabilizing interactions with strand 1 from domain 1 unfolds first. If the mechanical stability of the interdomain interface (between GroES domains 6 and 7) is higher than that of the intradomain interaction, then mechanical unfolding of the seventh GroES domain may involve the detachment and unfolding of strands 2–9 at low force. The next domain, which has not been destabilized by rupture of the interface involving its C-terminus, unfolds at a high force together with strand 1 of the adjacent domain, which is consequently destabilized, unfolding at a low force (similar to the first unfolding event). Unfolding of (GroES)$_7$ thus proceeds by repetition of this sequence, peeling domains off the domelike native structure, which is maintained (depicted in Fig. 4 a, left), giving rise to the alternation of the unfolding force and contour length values. The polarity induced in otherwise identical GroES domains by rupture of the interface between the 1st and 7th domain thus leads to two distinct mechanical resistant units (unfoldons) within GroES. Unfoldon 1 (low force resistance) comprises strands 2–9 ($\Delta L_c = 26.62$ nm) and unfoldon 2 (high force resistance) comprises stand 1 from the previous domain and strands 1–9 from the next domain ($\Delta L_c = 33.76$ nm, taking the length of the (Gly)$_1$ linker into account). It is noted that unfolding may also proceed from the N-terminus (Fig. 4 a, right). In this case, unfoldons 1 and 2 are likely to comprise the same components, but unfolding proceeds by the unfolding of domain 1 with an unstructured N-terminal $\beta$-strand (strand 1). This may unfold easily at low force to give a short $\Delta L_c$. The second subunit, which retains strand 1, is relatively stable and unfolds completely together with the first strand of the third subunit, giving a long $\Delta L_c$ and high unfolding force. The third domain, without the first strand, may unfold as described for domain 1 at low force, giving a short $\Delta L_c$, and domain 4 will unfold similarly to domain 2 etc. The similarity in predicted values of $\Delta L_c$ for each unfoldon in each unfolding scenario precludes identification of the actual unfolding pathway.

The force-induced deformation of the native domelike structure of sc(GroES)$_7$, together with the stepwise change in the end-to-end length of the remaining folded structure

**FIGURE 4** (a) Schematic representation of the suggested mechanical unfolding pathway of sc(GroES)$_7$, where we assume that unfolding proceeds from either the C-terminus (left) or the N-terminus (right; see text). The first and last $\beta$-strands of adjacent domains, whose interaction stabilizes the oligomeric form, are depicted as red and green rectangles, respectively. (b) Force-extension profiles from simulations of the individual unfoldons. Unfoldons extended at a constant velocity (0.01 $\text{Å}$/ps) using a cantilever with elastic constant of 10 pN/Å. Unfoldon 2 unfolds at a higher force, but the rate-limiting step is the same as for unfoldon 1. The small peak observed for unfoldon 2 at an extension of ~40 Å corresponds to the detachment of strand 1, which has no influence on the mechanical stability of the unfoldons. Structures are taken from the start of simulation (left, above the force-distance trajectories) and at the transition state (right).
and the alternation of the type of unfolding event (unfoldon 1 or 2) convolute to give the observed complex pattern of $\Delta L_e$ as a function of event number (Fig. 2 f). It is important to point out that values for $\Delta L_e$ calculated assuming the boundaries of unfoldons 1 and 2 and taking into account the effects described above (Fig. 2 f, dashed red line) shows a general agreement with those measured (Fig. 2 f, black line).

Simulating the forced unfolding of unfoldon 1 and unfoldon 2 demonstrates that each unfolds at considerably different forces. The force-extension profiles obtained by extending energy-minimized and equilibrated structures of the two unfoldons are shown in Fig. 4 b. Only the first peak is shown, since additional features of the force-extension profiles are not comparable with the experiment. The shape and position of the peaks suggest that the main unfolding event is essentially the same for the two unfoldons: the peak for unfoldon 2 occurs at an extension corresponding to that for unfoldon 1 plus the length of a fully extended strand 1. Although strand 1 extends first in unfoldon 2, the crucial events occur at the C-terminus: the rate-limiting step for unfolding corresponds to the breaking of the contacts between strands 6 and 9 and 1’ (the first strand of the next domain). For unfoldon 1, the sequence of events is the same, with the difference that strand 1’ is missing; this makes the sheet formed by strands 6 and 9 weaker, and thus, unfoldon 1 as a whole is weaker than unfoldon 2.

By utilizing a protein L/sc(GroES)$_7$ heteropolypeptide we have found that the mechanical strength of the GroES monomers within the (GroES)$_7$ heptamer varies as a function of event number. The alternating behavior of contour-length increase and unfolding force indicates that alternate unfolding events comprise not only a GroES monomer but an accompanying part of the next folded domain, which lowers the force required to unfold the remnants of the folded domain. The fact that this effect is apparent across the unfolding profile of the (GroES)$_7$ heptamer suggests that GroES multimers must retain a native-like structure. Although the relatively simple model based on two unfoldons adequately accounts for alternating force and $\Delta L_e$ values, it does not account for the observed apparent respective increase and decrease in unfolding forces and $\Delta L_e$ values for unfoldon 2 and unfoldon 1 as unfolding proceeds. These effects are challenging to address by both experimental and theoretical methods and may arise as a consequence of the complex changes in pulling geometry upon the stepwise destruction of the native domelike structure of sc(GroES)$_7$.

The mechanical properties of many proteins have been reported in the past 15 years. Most proteins studied so far are topologically simple and unfold in a single step (7,38). By contrast, multidomain proteins may unfold by a multistep pathway, and unfolding elements (or unfoldons) need not correspond to the individual domains but may involve more than one domain. A combination of experiment and simulation has recently demonstrated that interdomain interactions confer mechanical resistance and dominate the mechanical unfolding pathway of an ankyrin repeat protein (39). Here, we have shown that a homopolyprotein obtained by linking the individual domains in a naturally occurring complex can display a rich mechanical phenotype (24,25,29) by tuning the relative strength of intra- and interdomain contacts across identical domains.

**SUPPORTING MATERIAL**

A figure is available at http://www.biophysj.org/biophys/journal/supplemental/S0006-3495(12)00387-6.

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