Molecular origin of the extreme mechanical strength of an ester bond containing pathogen surface protein

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Abstract

Gram-positive bacteria can resist large mechanical perturbations during their invasion and colonization by secreting various surface proteins with intramolecular isopeptide or ester bonds. Previous studies showed that the isopeptide bond containing proteins are inextensible under mechanical load. However, compared to isopeptide bonds, ester bonds are prone to hydrolysis; it remains elusive whether ester bonds play similar roles as isopeptide bonds to completely block mechanical extension, or alternatively dissipate mechanical energy by bond rupture. Here, we show that an ester bond containing protein, the C1 domain of Cpe0147, is not extensible even at forces > 2 nN. The ester bond locks the structure to a partially unfolded conformation, in which the ester bond remains largely water inaccessible. This allows the ester bond to withstand considerable mechanical forces and in turn prevent complete protein unfolding. Inspired by this design principle, we engineer a disulfide mutant resistant to mechanical unfolding under reducing conditions.

Introduction

Gram-positive bacteria produce a variety of surface adhesion proteins for host surface binding, biofilm formation, and immune evasion^{1–6}. These proteins include rod-like pili and other large multi-domain proteins such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)^{7,8}. Despite that these proteins have distinct synthesis pathways, they share similar ‘beads on a string’ extended organization. Many of the domains in these proteins adopt Ig-like structure with unusual intramolecular covalent cross-links^{9,10}, including thioester^{11–13}, isopeptide^{14,15}, and ester bonds¹⁶. These bonds endow the proteins outstanding mechanical functions for strong surface anchoring or resisting large mechanical perturbations. Depending on the chemical reactivity and the location in the protein structures, thioester and isopeptide bonds have distinct mechanical functions. Isopeptide bonds are generally formed between the side chains of Lys and Asn (or Asp) residues and are chemically inert under physiological conditions^{9,17–20}. Both single molecule force spectroscopy experiments^{15,21,22} and molecular dynamics simulations²³ revealed that isopeptide bond containing proteins can withstand considerable mechanical forces. For example, spy0128, the major pilin subunit of *Streptococcus pyogenes*, is inextensible up to 800 pN due to the presence of isopeptide bond between the first and the last β strands¹⁵. Moreover, as the isopeptide bond is located at the site of stress concentration, it locks the protein in a partially unfolded structure and allows fast refolding under load²³. In another pilin subunit SpaA from *Corynebacterium diphtheriae*, the isopeptide bond can also restrict the unfolding of this CnaA Ig-type domain from complete unfolding under mechanical stress²². Unlike isopeptide bonds, thioester bonds are more labile and can react with amino groups that are abundant in host tissues¹². They are often present at pilin tip-end adhesins and are formed in between the side chains of Cys and Gln residues. The reaction of thioester with nucleophilic ligands creates strong covalent adhesion with the host surfaces to resist large mechanical stresses¹². Interestingly, recent studies revealed that the reactivity of thioester bonds is high at low forces and can be completely blocked at forces larger than 35 pN, entailing the bacteria stress dependent mobility²⁴.

Ester bond is another type of intramolecular covalent bonds discovered in Gram-positive surface proteins and is formed between the side chains of Thr and Gln residues^{9,16,25}. It was first discovered in a surface-anchoring protein Cpe0147 from *Clostridium perfringenes* and then widely found in many bacterial surface adhesion molecules. The ester bonds in those proteins generally locate at the same position as the isopeptide bond in spy0128. However, as ester bonds are prone to hydrolyze at both acidic and basic conditions²⁶, it is largely unknown how such unique chemical properties affect the mechanical properties of ester bond containing proteins. Previous studies showed that Cpe0147 is resistant to hydrolysis at pH 9 and in the presence of 6 M urea, implying that the ester bond is shielded from the attack of OH⁻ ions²⁵. However, as mechanical force may alter the structure of this protein locally, it remains elusive whether force can alter the reactivity of the ester bond and cause distinct mechanical responses. Understanding the mechanical response of the ester bond containing proteins may provide new insights into the pathological mechanism of Gram-positive bacteria and help to develop antibiotics based on blocking the formation of ester bonds.

In this work, we employed atomic force microscopy-based single-molecule force microscopy^{27–32}, protein engineering, and molecular dynamics simulation to study the mechanical properties of the C1 domain of Cpe0147. We found that C1 exhibited similar mechanical stability as the isopeptide bond containing protein spy0128 and was inextensible even under forces > 2 nN. In contrast, if force was applied from a different orientation to unfold the protein structure, the ester bond broke at forces of only ~ 80 pN. These results suggested that the interplay between the ester bond and the protein structure is critical to the mechanical properties of the C1 domain. Molecular dynamics simulations revealed that the ester bond locked the structure to a partially unfolded conformation, in which the ester bond remained largely water inaccessible. This allowed the ester bond to withstand considerable mechanical forces and in turn to prevent complete protein unfolding. Such a coupling between the ester bond and the protein structure was also found in another ester containing proteins, ParV of *Parvinmonas sp.*. Even the ester bond was replaced by a disulfide, the mechanical stability of the protein was not affected. Taken together, our studies revealed an uncharted mechanism to allow adhesion proteins to withstand extreme mechanical load using weak bonds.

Results

Ester bond containing C1 domain is inextensible under mechanical forces > 2 nN

Cpe0147 is a multiple-domain protein that covalently links the bacterial cell wall and the adhesin¹⁶ (Fig. 1a). The domains in the stalk region adopt similar all β -strand IgG-like fold and contains an ester bond linked the Thr on the first and the Gln on the last β -strands¹⁶ (Fig. 1b and c). To investigate the mechanical response of Cpe0147, we used the wide type C1 domain (C1_{WT}) as the model system. We engineered a chimeric polyprotein, Fg β -(GB1)₂-C1_{WT}-(GB1)₂-cys for single molecule atomic force microscopy (AFM) experiments (Fig. 1d), following the experimental protocol reported by Milles et al.²⁷.

This polyprotein features specific noncovalent/covalent linkage of the polyprotein to either the cantilever tip or the substrate as well as mechanical fingerprint units for unambiguously identifying single molecule events. The proteins were covalently linked to the substrate through the thiol group of the C-terminal Cys and then picked up by the SdrG modified cantilever tip through strong noncovalent interactions between Fg β and SdrG (rupture forces > 2 nN)^{28,33}. Each end of C1_{WT} was flanked with two GB1 domains. The mechanical unfolding of GB1 characterized by a contour length increment of ~ 18 nm and an unfolding force of ~ 200 pN at a pulling speed of $1.6 \mu\text{m s}^{-1}$ ³⁴⁻³⁶. Stretching the polyprotein allowed us to apply forces to C1_{WT} between its N- and C-termini, similar to the force direction the protein domain experiences in their biological settings.

Stretching polyprotein led to representative sawtooth-like force-extension curves (Fig. 1e), in which each sawtooth peak corresponds to the force-induced unfolding of an individual domain in the polyprotein chain. However, we only observed five peaks; four of them showed the same contour length increments of 18 nm, which can be attributed to the unfolding of the four GB1 domains. The last peak of more than 2 nN arised from the unbinding of the Fg β /SdrG interaction²⁸. There was no other peaks observed, indicating that C1_{WT} did not fully unfold even under pulling forces higher than 2 nN. We intended to further increase the maximum forces applied to C1_{WT} by using covalent linkages to anchor the polyproteins to both the cantilever tip and the substrate. We constructed another polyprotein, Spytag-(GB1-C1_{WT})₄-cys. Spytag can bind Spycatcher to form a covalent isopeptide bond^{37,38}. Unfortunately, the maximum detaching force in this design was even lower than that based on the Fg β /SdrG interaction, presumably due to the maleimide-thiol chemistry we used for protein immobilization, which was not strong enough if the conjugate was not hydrolyzed³⁹. In this covalent linking scheme, each molecule can be stretched only once, preventing the mechanically triggered hydrolysis of maleimide-thiol adducts³⁹. Nonetheless, we only observed the unfolding events from four GB1 domains using this polyprotein (Supplementary Fig. 1). It is safe to conclude that the wild type C1 domain from Cpe0147 is inextensible up to the forces that can break weak covalent bonds.

Mechanical stability of the C1 domain without ester bond

To illustrate the role of ester bond in maintaining high mechanical stability of the C1 domain, we designed a protein variant C1_{T11A} in which the ester bond was eliminated by mutating ester bond forming Thr at the 11th position to Ala (Fig. 2a and b). Also, the polyprotein Fg β -(GB1)₂-C1_{T11A}-(GB1)₂-cys was constructed and then studied using AFM-based single molecule force spectroscopy. In the force-extension curves, besides the four peaks of ΔLc of ~ 18 nm for GB1, we observed an additional peak of ΔLc of 47.4 ± 3.25 nm (colored in red) (Fig. 2c). The contour length increment is consistent with the complete mechanical unfolding of C1 ($\Delta Lc = 146 \text{ aa} \times 0.365 \text{ nm/aa} = 5.2 \text{ nm}$). The unfolding forces of C1_{T11A} domain were about 92 ± 41 pN (Fig. 2e), which were lower than that of GB1. Eliminating the ester bond by the T11A mutation significantly reduced the unfolding forces of C1_{WT}. Therefore, the ester bond plays an important role in stabilizing the wild type C1 structure under mechanical load.

Ester bond is mechanically weak in the absence of the folded C1 structure

To further understand the contribution of ester bond to the mechanical stability of C1, we design a circular permutant of C1 ($C1_{CP}$) to structurally decouple the rupture of ester bond and the unfolding of $C1_{WT}$. In $C1_{CP}$, the N- and C- termini of C1 were connected by an elastin like peptide (ELP) of 45 amino acids, and the protein was split between the residues 125 and 126 to form the new N- and C- termini (Fig. 3a and b). Previously, Young et al. have shown that the two fragments obtained by splitting $C1_{WT}$ at this position can spontaneous rebind and form the inter-molecular ester bond²⁵. Therefore, we anticipated that the ester bond can also formed in $C1_{CP}$. Note that in this new pulling direction, the ester bond is not located at the force concentration point anymore. We would expect to see the rupture of ester bond after the unfolding of $C1_{CP}$, providing an unambiguously way to quantify the strength of the ester bond in the absence of the folded protein structure.

$C1_{CP}$ was stretched in the polyprotein $Fg\beta-(GB1)_2-C1_{CP}-(GB1)_2-cys$ construct and the representative force-extension curve is shown in Fig. 3c. In addition to the four peaks whose ΔLc correspond to the unfolding of GB1, we observed two additional peaks of ΔLc of 38 ± 1.2 nm (colored in green) and 23 ± 0.5 nm (colored in orange). The event in green always occurred first and then followed by the event in orange. Matching to the protein structure, these two events can be assigned to the piecewise extension of $C1_{CP}$ (Fig. 3d). The first peak corresponded to the unfolding of the protein and then extending the peptide sequence outside the ester bond (Thr-Gln). The measured ΔLc matches well with the theoretically calculated value of ~ 39 nm ($((110-12) + (140-129)) \text{ aa} \times 0.365 \text{ nm/aa} - 0.9 \text{ nm} + 0.6 \text{ nm}$), where 0.9 nm is the distance between V113 and Q129 and 0.6 nm is the length of the ester bond containing linkage between T11 and Q141 (Fig. 3e). The unfolding forces of $C1_{CP}$ were 91.6 ± 52 pN (Fig. 3f). The second peak resulted from the force-induced rupture of the ester bond and subsequently extending the peptide sequence shielded by this bond, as the measured ΔLc of 23 ± 0.5 nm matched well with the theoretically calculated value of 23 nm ($66 \text{ aa} \times 0.365 \text{ nm/aa} - 0.6 \text{ nm}$) (Fig. 3g). The forces for the rupture of the ester bond were only 77 ± 56 pN (Fig. 3h), much lower than that of typical covalent bonds⁴⁰⁻⁴³ and even some non-covalent bonds⁴⁴⁻⁴⁷. We inferred that this may be because the rupture of ester bond in water undergoes a hydrolysis mechanism²⁶, distinct from the free radical mechanism for the rupture of other covalent bonds⁴⁸. Nonetheless, these results clearly indicated that an unprotected ester bond cannot provide sufficient mechanical strength to establish strong adhesion. Since we did not observe any ester bond hydrolysis events in $C1_{WT}$, we proposed that the ester bond remained shielded from water attack even $C1_{WT}$ was stretched to forces higher than 2 nN.

Molecular dynamics simulations

To understand the molecular details underlying the ultra-high mechanical stability of C1, we performed steered molecular dynamics simulations for both $C1_{WT}$ and $C1_{CP}$ (Fig. 4a) (See Methods for more

details). For C1_{WT} without force load, the ester bond was buried to a large extent and shielded from water attack by the nearby residues (Fig. 4b). When a constant pulling force of 1500 pN was applied to the N- and C-terminal residues, the protein structure only partially unfolded at the force bearing first and last β -strands. However, as the ester bond was located at the force-concentrating point, it prevented force propagation to the rest parts of the protein and made the partially unfolded structure resemble the native structure (Fig. 4c). As the rupture of the ester bond requires water attack, the structural integrity of C1_{WT} can in turn ensure high mechanical stability of the ester bond. In comparison, for C1_{CP}, the ester bond started to sustain the pulling force only at the later stage of the unfolding event. Applying pulling force led to complete protein unfolding and exposure of the ester bond to water (Fig. 4d). To estimate the reactivity of the ester bonds in the three conditions, we quantified the number of water molecules within 5 Å from the O ϵ 1 atom of the Gln141. For C1_{WT}, even at a constant pulling force of 1.5 nN, the number of water molecules accessible to the ester bond was only slightly higher than that without force load (Fig. 4e). In contrast, for C1_{CP}, the ester bond was surrounded by $\sim$ 9 water molecules due to the unfolding of the protein structure. Detailed structural analysis revealed that the bulky side chains of residues K10, T12, H133, D138, and A140 near the ester bond form a cage to shield the water molecules outside (Supplementary Fig. 2a). This cage structure was well maintained in C1_{WT} even under a stretching force of 1.5 nN and only slightly open at 2 nN, as indicated by the root-mean-square deviation (RMSD) of these residues (Supplementary Fig. 2b). However, in C1_{CP}, this cage fell apart quickly, making the ester bond exposed to water molecules. Moreover, without the ester bond, even in C1_{WT}, the residues that form the ester bond were exposed to water molecules within a few hundreds of picoseconds (Supplementary Fig. 2c). The maximum number of water molecules was $\sim$ 14, which was even higher than that for C1_{CP} with the ester bond under forces. Comparing the distance evolution between the ester bond forming residues (Supplementary Fig. 2d) and the overall structural change of the protein in the absence of ester bond (Supplementary Fig. 2e), we can clear see that the separation of the ester bond forming residues was always prior to the increase of RMSD, indicating that breaking of the ester bond is a necessary step toward complete unfolding of the overall protein structure. Such results clearly demonstrated the mutual interplay between the chemical event and protein mechanical stability.

C1 structure as a “safe house” to protect mechanical rupture of disulfide bonds

Inspired by the molecular mechanism that allows the integration of labile ester bond and weak protein fold to achieve remarkable mechanical stability, we hypothesized that it is also possible to use the C1 structure as a mechanically robust cage or “safe house” to prevent other chemically active bonds. To this extend, we replaced the ester bond in C1 to disulfide bond^{49,50} through T11C and Q141C mutation (the protein is named as C1-df hereafter), and studied the mechanical stability of this protein in the presence of reducing agent (10 mM tris (2-carboxyethyl) phosphine, TCEP). We built a polyprotein Fg β -(GB1)₂-C1-df-(GB1)₂-Spytag and linked it to the cys-GB1-Spycatcher modified substrate by using Spycatcher/Spytag interaction instead of direct thiol-maleimide interaction to avoid the formation of mismatched disulfide

bond that otherwise would complicate the single-molecule AFM experiments. The experimental scheme is shown in Fig. 5a. Note that the disulfide bond was not 100% formed in all proteins. Thus, the mechanical unfolding of C1-df could result in three different scenarios (Fig. 5b): (1) The unfolding of C1-df without the disulfide bond (denoted as the “Disulfide unformed” group); (2) The complete locking of C1-df with disulfide bond at the folded state without unfolding (denoted as the “Disulfide unruptured” group); (3) The hydrolysis of the disulfide bond in C1-df and the release of the sequestered peptide sequences upon stretching (denoted as the “Disulfide ruptured” group). The representative force-extension curves of the three groups are shown in Fig. 5c-e. Unlike the case of ester bond, which forms in all C1 domains, the disulfide bond is only formed in 86.7% of the total events (168 out of 193) (Fig. 5f). Among them, the “Disulfide ruptured” events only account for 4.3% (16 out of 389) of the total events (Fig. 5f). However, in the absence of reducing agent, this group is missing. This indicates that the disulfide bond in C1-df is also protected from the attack of the reducing agent. To further confirm this, we studied the mechanical unfolding of the circular permutant of C1-df (C1_{CP}-df), by replacing the ester bond in C1_{CP} to disulfide bond (Fig. 6). As in C1_{CP}-df, the partially unfolded protein structure cannot prevent the attack from TCEP, the rupture of disulfide bonds was observed in 42.7% of the total events. Even without TCEP, in 3.1% of the traces, we observed the rupture of disulfide bonds at forces of ~ 2 nN. Taken together, our results indicated that the C1 structure can efficiently prevent the reducing of the imbedded disulfide bond in the reducing environment under forces, similar to the protecting effect for the ester bond in C1_{WT}.

Note that the ΔL_c of the “Disulfide ruptured” group is ~ 41 nm, which is shorter than the ΔL_c of the “Disulfide unformed” group (~ 47 nm). This suggests that while the C1-df with the disulfide bond can withstand significant stretching forces, it is partially unfolded to the disulfide bond position at high forces with a ~ 6 nm shortening of the contour length. However, in these events, we did not observe the transition from the folded state to the partially unfolded state in the force-extension curves, which may indicate that C1-df is already at the partially unfolded state prior to the measurement. Introducing the disulfide bond may reduce the thermodynamic stability of the protein, making the protein occasionally adopt the unfolded conformation even the disulfide bond is formed in the protein structure. In contrast, wild type C1 cannot spontaneously unfold to make the ester bond solvent accessible due to its high thermodynamic stability.

Discussion

By employing single molecule force spectroscopy, protein engineering, and molecular dynamics simulation, we studied the nanomechanical properties of the C1 domain of Cpe0147 from *Clostridium perfringens* with a spontaneously formed intramolecular ester bond. Our results revealed that C1 can retain a partially unfolded structure under a force as high as 2.5 nN. When the ester bond was removed, the mechanical stability of the protein C1_{T11A} dropped sharply with unfolding forces of ~ 92 pN. This demonstrates the important role of covalent ester bond for the mechanical stability of C1. However, using a circular permutation of C1 (C1_{CP}), we revealed that the protein can first partially unfold at a force of ~ 90 pN and then rupture the ester bond at a force of ~ 80 pN. Although all intramolecular interactions are

kept in C1_{CP}, the change of the force orientation can dramatically affect the mechanical response. These results highlight the importance of the interplay of the protein structure and the ester bond to the ultra-high mechanical stability: The ester bond locates at the force concentration point to lock the protein structure to a partially unfolded state with most of the native contacts preserved. On the other hand, the protein structure prevents the ester bond from water attack and makes this chemically labile bond mechanically stable. This stabilization mechanism was verified using molecular dynamics simulation. Moreover, using disulfide bond mutants, we showed that the unique correlation of the protein structure and the location of the intramolecular bond is responsible to the ultra-high mechanical stability of the protein domain, regardless what type of bonds at the force concentration point. This can also explain that the ester bond containing proteins show similar mechanical properties as the isopeptide bond containing proteins with similar structures.

Previous bioinformatics analysis suggested that this type of ester bonds is conserved in many cell surface proteins of Gram-positive bacteria. Moreover, these ester bond containing proteins share similar structures despite that they show very low sequence homology¹⁶. We hypothesized that these proteins also exhibit outstanding mechanical stabilities. To confirm this, we studied the mechanical unfolding of another ester bond containing protein ParV from the Gram-positive bacterium, *Parvimonas sp.* (Supplementary Fig. 3a and b). Similar to the C1 domain of Cpe0147, we did not observe any unfolding signatures of wide type ParV up to a stretching force of 2 nN (Supplementary Fig. 3c). Once the ester bond was deleted by mutation, the protein unfolded at forces of ~ 100 pN (Supplementary Fig. 3d). Therefore, we propose that the chemically labile ester bonds play similar roles as isopeptide bonds to stabilize the structure of Gram-positive surface proteins. In contrast, the thioester containing surface proteins exhibit distinct structures and the position of the intramolecular bond. They are likely to provide different mechanical functions, such as anchoring to host surfaces¹².

Why has Cpe0147 evolved such exceptional mechanical stabilities? As one of the major putative MSCRAMMs of *Clostridium perfringens*, it anchors to the cell wall through its C-terminus and projects its N-terminal adhesion domain through 11 repeat ester bond containing Ig domains. The internal ester bonds are strategically positioned to covalently link the first and last β -strands of these Ig domains⁹. Therefore, the stress on the proteins is only propagated from the first β -strand, through the ester bond, and to the final β -strand (β 7), leaving the rest of the protein bypassed by the mechanical forces. Mechanical unfolding of these domains may affect the self-assembly and function of these domains or even make them susceptible to proteolytic attack. Their extremely high mechanical stability allows them to maintain folded structures even under mechanical forces of more than 2 nN, which is critical to their biological functions.

It remains unknown whether Cpe0147 can bind to the host or other cell adhesion proteins via the ester bond containing stalk region⁵¹. If so, we hypothesize that the force direction can be altered by such binding events and eventually cause the local unfolding of the protein structure and expose the ester bond to water, similar to the circular permutant of C1 studied in this work. This can lead to the hydrolysis

of the ester bond, then complete unfolding of these domains, and eventually loss of adhesion. In contrast, the isopeptide bond containing proteins can only partially unfold in this scenario and refold back after the release of force due to the high stability of isopeptide bonds.

Another interesting feature of C1 is that it contains two calcium binding sites¹⁶. Previous biochemical studies showed that these calcium binding sites do not contribute to the thermodynamic stability or the ester bond formation²³. It was proposed that they may change the local structural flexibilities. It is plausible that calcium ions can serve as a gate to control the water accessibility of the ester bond especially at the partially unfolded state under stress. Further experimental work is required to verify this hypothesis.

Conclusion

In summary, we employed atomic force microscopy-based single-molecule force microscopy, protein engineering, and molecular dynamics simulation to study the mechanical properties of an ester bond containing protein, the C1 domain of Cpe0147 from Gram-positive bacterium, *Clostridium perfringens*. We find that despite that individual ester bond is mechanically much weaker than an isopeptide bond, C1 exhibits similar mechanical stability as the isopeptide bond containing protein and does not completely unfold even under forces > 2 nN. The ester bond locks the structure to a partially unfolded conformation, in which the ester bond remains largely water inaccessible. This allows the ester bond to withstand considerable mechanical forces and in turn prevent complete protein unfolding. Breaking this structural correlation by circular permutation leads to sequential protein unfolding and ester bond hydrolysis. We further highlight the importance of the position of ester bond on the extreme mechanical strength of the protein by engineering a mutant with a disulfide bond at the ester bond location that can retain high mechanical stability in the presence of reducing agents. These results provide the molecular mechanism on the extreme mechanical stability of ester bonding containing protein and may also inspire the design of new antibiotics by mechanically destabilizing these proteins.

Methods

Chemicals. All chemicals used were supplied by Sigma-Aldrich (USA) or New England Biolabs (USA) if not specified explicitly.

Gene construction. The C1 domain of Cpe0147 from *Clostridium perfringens* (GenBank accession no. EDT23863.1), the *Staphylococcus epidermidis* SdrG N2 and N3 domain genes, as well as GB1 were synthesized codon-optimized for expression in *Escherichia Coli* as linear DNA fragments with suitable overhangs (GenScript, China). Genes were cloned into pET22b or pQE80L Vectors. The T11A mutant and circular permutation were introduced by blunt end ligation cloning using T4 Ligase. Final open reading frames of all constructs were checked by DNA sequencing (GenScript, China). The complete sequences of all protein constructs used are listed in Supplementary Information.

Protein expression and purification. The genes encoding the proteins used in this article were synthesized and codon-optimized for expression in *Escherichia coli* (BL 21) cells. Precultures of 5 mL in LB medium (BD Difco, USA) containing 100 µg/mL ampicillin (Macklin, China), grown overnight at 37 °C, were inoculated in 500 mL LB medium containing 100 µg/mL ampicillin and grown for 3 h at 37 °C and then induced with 0.4 mM of IPTG (Aladdin, China) overnight at 25 °C. Bacteria were harvested by centrifugation at 10000 g, and pellets were stored frozen at -80 °C until purification.

All purification steps were performed at 4 °C to 8 °C. The bacterial pellet was resuspended in PBS, the cells were then lysed through mechanically sonication (ATPIO, China) followed by centrifugation at 11000 g for 1 h. The His₆-tagged proteins were purified by affinity chromatography using a Talon column (Sigma-Aldrich, USA). The supernatant was washed extensively and then eluted in the same buffer supplemented with 200 mM imidazole. Protein containing fractions were concentrated in the centrifugal filters, exchanged into measurement buffer by desalting columns (Sigma-Aldrich, USA), and frozen in aliquots in liquid nitrogen to be stored at -80 °C until used in experiments. Protein concentrations were measured by spectrophotometry at 280 nm with typical final concentrations of ~ 100 µM (Thermo Nanodrop 2000, USA).

AFM sample preparation. More detailed AFM-based single molecule force spectroscopy protocol have been published previously^{28,39}. In brief, AFM cantilevers (Bruker, MLCT, USA) and glass surfaces (SAIL BRAND, China) were modified with aminosilane.

Glass surface: Glass substrates were cut into 1 × 1 cm² slides, soaked in a chromic mixture overnight, thoroughly washed with deionized (DI) water, ethanol and acetone successively, and then dried under a stream of nitrogen to produce surfaces with exposed hydroxyl groups. These substrates were immersed in an anhydrous toluene solution containing 1% (v/v) APTES (Merck, USA) at room temperature (R.T.) for 1 h for amination. Then, they were washed with toluene and ethanol, dried under a nitrogen flow. Finally, surfaces were baked at 90 °C for 30 min. Glass substrates were stored in a desiccator under Argon and typically used within half month.

Cantilevers: Silicon nitride (Si₃N₄) cantilevers (MLCT-D, Bruker) were first cleaned with Milli-Q water, and then placed in a chromic mixture (chromic acid) at 80 °C for 30 min. After that, the cantilevers were washed with water, then ethanol, and dried under a stream of nitrogen. Then the hydroxylated cantilevers were immersed in 1% (v/v) APTES in toluene for 1 h. After that, they were rinsed with toluene, then ethanol, dried under a nitrogen stream, and incubated at 80 °C for 45 min. Finally, they were stored overnight under Argon and used in the following steps the next day.

Both glass substrates and cantilevers were immersed in DMSO containing 0.2 mM Mal-PEG-NHS (MW: 5000 Da, Nanocs, USA) for 1 h. After being washed with DMSO, ethanol and dried under a nitrogen stream, the resulting Mal-coated glass substrates and cantilevers were kept dry at -20 °C for use in the following protein modification steps in single-molecule experiments.

AFM-based single molecule force spectroscopy. The force spectroscopy experiments were carried out using a commercial JPK ForceRobot 300 AFM system (JPK Instruments AG, Germany). Experiments were conducted at room temperature (22 °C) and performed in 10 mM PBS buffer with or without 10 mM TCEP if needed. Soft silicon nitride MLCT-D cantilevers of typical spring constant of 30 ~ 40 pN nm⁻¹ were used for all experiments and calibrated using the thermal tune method after allowing the cantilever to equilibrate in solution for at least 30 min. Cantilevers were briefly and gently (~ 300 pN) brought in contact with the functionalized surface and held at the surface for 0.5 s, then retracted at constant velocity of 1.6 μm s⁻¹. The force-extension curves were recorded using JPK data processing software and were further analyzed by a custom-written procedure in Igor 6.12 (Wavemetric, Inc).

Molecular Dynamics Simulations. The molecular dynamics simulations were conducted by the GROMACS software⁵² with the ff14SB force field⁵³ and TIP3P water⁵⁴. To model the ester bond, we introduced a covalent bond between the Thr-11 (Oγ1) and Gln-141 (Cδ). The extra atoms (Hy1 of Thr-11; Nε2, Hε21, and Hε22 of Gln-141) were removed, with their partial charges being integrated into the nearby heavy atoms. The atomic coordinates of the C1_{WT} were taken from the Protein Data Bank (entry 4MKM)¹⁶. The five N-terminal residues lacking structural information were not included. In constructing the C1_{CP}, a ELP linker with the length of 20 amino acids was added between the two termini of the C1, and the residues Leu-125 and Asp-126 were used as the new termini. The three-dimensional structure of the linker was modelled by the ModLoop^{55,56}.

The C1_{WT} and C1_{CP} were solvated in the rectangular water boxes with the dimensions of ~ 241 Å×84 Å×85 Å (with 48891 water molecules) and 1017 Å×74 Å×74 Å (with 159145 water molecules), respectively. Sodium ions were added to neutralize the systems. The LINCS algorithm was used to restrain the covalent bond involving hydrogen atoms⁵⁷. After a 50000-step minimization using the steepest descent method, each system was equilibrated for 0.1 ns in the NVT ensemble and another 0.1 ns in the NPT ensemble. The temperature and pressure were controlled at 298.0 K and 1.0 atm, respectively. The heavy atoms of the proteins were restrained to their original positions by a harmonic potential during the minimization and equilibrium steps. Starting from the equilibrated structures, we performed steered MD simulations by applying pulling force between the termini residues along the x-axis in NVT ensemble. For the C1_{WT}, the constant pulling force were applied to the termini residues with the strength of 1500 pN and the simulations were lasted for 10 ns. For the C1_{CP}, we firstly conducted a constant velocity pulling simulations with the pulling speed of 49 Å/ns, such that the ester bond starts to sustain pulling force. Then we performed the same constant pulling force simulations as that in the C1_{WT}. Two independent simulations were conducted for each system. To increase the statistics, we also performed another two (one) constant pulling force simulations with the length of 35 ns for the C1_{WT} (C1_{CP}). The snapshots sampled during the constant pulling force simulations were used for analysis. The software PyMOL was used for the structure visualization.

Declarations

Competing interests

The authors declare no competing interests.

Author contributions

Y. C., H. L. and W. W. conceived the project and designed the experiments. H. L., Q. M. performed all the experiments and analyzed the data. W. L. designed and performed the simulations. J. W. and H. M. performed the quantum mechanics calculation of ester bond hydrolysis. Y. C., W. W. and M. Q. supervised the project. H. L. and Y. C. wrote the paper with contributions from all authors.

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Data availability

The data that support the findings of this study are available from the corresponding authors upon request.

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Figures

Figure 1

Cpe0147 is extremely resistant to mechanical forces. **a**, Cpe0147 links the tip adhesion domain and bacteria to establish invasion and colonization and experiences considerable mechanical forces in the biological settings. **b**, Structure of the C1 domain from PDB (4MKM). The ester bond is highlighted in magenta and the two calcium ions are shown as orange spheres. **c**, Topology of C1WT domain. The ester bond is formed between Thr11 and Gln141. **d**, Schematic of the AFM based single molecule force spectroscopy experiments. Fgβ-(GB1)₂-C1WT-(GB1)₂-cys was covalently anchored to a glass surface through a polyethylene glycol (PEG) linker via thiol-maleimide chemistry and picked up using a SdrG-cys modified cantilever. **e**, Mechanical extension of C1WT from its N- and C-termini is blocked by the ester bond as the ester bond is located right at the force concentration point. **f**, A representative single-molecule force-extension trace at 1.6 $\mu\text{m s}^{-1}$ showing the unfolding of the four GB1 domains (black arrow) at ~200 pN but no unfolding peak of C1WT up to the rupture forces of Fgβ-SdrG complexes (red arrow).

Figure 2

Mechanical unfolding of the mutated C1 domain without the ester bond. a, Thr11 of C1 was mutated to Ala to eliminate the ester bond to yield the C1T11A mutant. b, Topology of C1T11A. The ester bond cannot form in the mutant. c, Representative single-molecule force-extension curve of stretching Fg β -(GB1)₂-C1T11A-(GB1)₂-cys following the same experimental protocol shown in Fig. 1d. Each peak was fitted by worm-like chain (WLC) model of polymer elasticity. The peak with a ΔL_c of 47 nm corresponds to the unfolding of C1T11A, the next four peaks correspond to the unfolding of GB1 domains, and the last peak corresponds to the rupture of the Fg β /SdrG complex. d, Histogram of contour length increment for C1 unfolding is centered ~ 47 nm. e, Unfolding force histogram of C1 domain at a pulling speed of $1.6 \mu\text{m s}^{-1}$ measures an average unfolding force of 92 ± 41 pN ($n=129$).

Figure 3

Mechanical unfolding of the circular permutant of C1, C1CP. a, Structure of C1CP based on PDB (4MKM). b, Topology of C1CP. Ester bond formed between T11 and Q141 is highlighted in cyan. The new N- and C-termini are at the position 126 and 125, respectively. The original N- and C-termini are connected by an ELP loop. c, Representative force-extension curve of stretching (GB1)₂-C1CP-(GB1)₂ following the same experimental protocol shown in Fig. 1d. The force peaks with ΔLc of ~38 nm (green) and ~23 nm (orange) are assigned as the unfolding of C1CP and the rupture of ester bond, respectively. The force peaks of ΔLc of ~18 nm correspond to the unfolding of GB1 domains. d, Schematic illustration of the contour length change upon stretching C1CP. First, the protein unfolds up to the ester bond position, then the rupture of ester bond releases the sequestered sequence. e, Histogram of ΔLc for C1CP unfolding events peaks at 38 nm. f, Unfolding force histogram of C1CP centers at 91.5 ± 52 pN (n=243). g, Histogram of ΔLc corresponding to the rupture of ester bond in C1CP peaks at 23 nm. h, Histogram of the rupture forces of ester bond centers at 77 ± 56 pN (n=243).

Figure 4

Molecular dynamics simulations demonstrating different water accessibility of the ester bond in C1WT and C1CP under pulling force. a, Cartoon representation of the three-dimensional structure of C1WT under the pulling force of 1500 pN. The residues Thr11 and Gln141 forming the ester bond were also shown by sticks representation. b-c, Three-dimensional structure of C1WT shown by sphere representation without force (b) and under the pulling force of 1500 pN (c). d, Three-dimensional structure of C1CP under the pulling force of 1500 pN shown by sphere representation. For clarity, the zoom in structure was also shown. The residues Thr11 and Gln141 were colored in red in the panels b-d. e, Distribution of the number of water molecules within 5 Å from the Oε1 atom of the Gln141 for C1WT (blue) and C1CP (orange) at the constant pulling force of 1500 pN. For comparison, the result for C1WT (black) without applying force was also shown.

Figure 5

Mechanical unfolding of C1-df. **a**, Schematic of the AFM based single molecule force spectroscopy experiments. Polyprotein Fg β -(GB1)₂-C1-df-(GB1)₂-Spytag was linked to the cys-GB1-Spycatcher modified substrate covalently through the Spycatcher/Spytag chemistry and picked up by the SdrG-cys modified cantilever through the Fg β /SdrG interaction. Thus, the force-extension curves should contain five GB1 fingerprints. **b**, Three possible unfolding/hydrolysis pathways of C1-df under load and the corresponding contour length change. **c**, Representative force-extension curves of the "Disulfide

unformed" group. Two populations of unfolding events as showed, five peaks in black related to GB1 unfolding and the purple one with ΔL_c of 47 nm corresponds to the unfolding of C1-df. d, Representative force-extension curves of the "Disulfide unruptured" group. The traces show only five GB1 unfolding events without the signature of C1-df unfolding or disulfide bond rupture. e, Representative force-extension curves of the "Disulfide ruptured" group. Except for the five GB1 unfolding events, there is an additional peak with ΔL_c of 41 nm (colored in orange) corresponding to the rupture of the disulfide bond. f, Pie chart shows the relative populations of the three kinds of events without (left, n=193) or with (right, n=389) 10 mM TCEP.

Figure 6

Mechanical unfolding of C1CP-df. C1CP-df was constructed in the same way as C1CP except that the ester bond was replaced by a disulfide bond. a, Three possible unfolding/hydrolysis pathways of C1CP-df under load and the corresponding contour length change. b, Representative force-extension curves of the "Disulfide unformed" group. c, Representative force-extension curves of the "Disulfide unruptured" group. d and e Representative force-extension curves of the "Disulfide ruptured" group in PBS with (d) and

without (e) 10 mM TCEP. f, Histogram of the mechanical rupture force of disulfide bond in PBS with 10 mM TCEP. g, Pie chart shows the relative populations of the three kinds of events without (left, n=844) or with 10 mM TCEP (right, n=754).

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