**Isopeptide-Blocker Impairs the Mechanics of Recently Translated Pilin Proteins**

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**ABSTRACT**

Gram-positive bacteria, such as *Streptococcus pyogenes*, use their adhesive pili to attach to host cells during early stages of a bacterial infection. These extracellular hair-like appendages experience mechanical stresses of hundreds of picoNewtons; however, the presence of an internal isopeptide bond prevents the protein from unfolding. Here, we designed a peptide to intervene in the formation of the isopeptide bond on the pilin Spy0128 from *Streptococcus pyogenes*, preventing folding and rendering the Spy0128 susceptible to protease digestion. By using a combination of protein engineering and single-molecule force spectroscopy, we demonstrate that the isopeptide-blocker peptide interferes with the formation of the isopeptide bond. While the intact Spy0128 is inextensible under mechanical forces, the intervened Spy0128 is completely extensible and lacks of mechanical stability. We propose that this isopeptide-blocker affords a novel strategy for mechanically-targeted antibiotics which, by blocking the folding structure of bacterial pili, could prevent the colonization of infectious microorganisms.

**Keywords:** protein folding; isopeptide bond; protein mechanics; peptide antibiotic; single-molecule force spectroscopy.

**Running title:** Interfering with the mechanics of inextensible pili proteins.
INTRODUCTION

Human pathogens use multiple strategies to adhere themselves to the host cell (1). In bacteria, a specialized filamentous structure known as pilus, enable bacterial attachment (2). Whereas in Gram-negative bacteria pili are made of several proteins stabilized by hydrogen bonds and other non-covalent interactions (3), pili from Gram-positive bacteria are assembled as a single polyprotein where each protein subunit is covalently linked through an intermolecular isopeptide bond (4). This particular feature—covalently attached protein subunits—, allows Gram-positive bacteria, such as *Streptococcus pyogenes*, to withstand large mechanical stresses (5, 6). This coccoid bacterium is responsible for the common strep throat disease (7), but also for the more severe necrotizing fasciitis, or flesh-eating disease (8). The pilus of *S. pyogenes* is formed by three different proteins: Spy0125 or adhesin, which contains the binding domain to the host cell and is located at the end of the pilus; Spy0128 or shaft protein, which forms the rod of the pilus; and Spy130 or base, which anchors the entire micrometer-long filament to the cell wall (9) (Figure 1). The titanic size of the *S. pyogenes* pilus, which can have an extension that exceeds the diameter of the bacterium (10), is principally due to the structural shaft Spy0128 protein. With more than 100 repeats along the pilus, each Spy0128 has a molecular weight of ~35 kDa and is made of two immunoglobulin-like fold domains arranged in a tandem manner (11) (Figure 1 and S1). Nevertheless, the mechanical stresses are transmitted by the C-terminal domains only (11), since the carboxyl terminal forms a peptide bond with the side chain of Lys161 of the next subunit, leaving the majority of the N-terminal domain outside of the pulling vector (Figure S1).

Upon bacterial adhesion, the pili rod domains withstand mechanical stresses that can reach several nanoNewton (5), forces that would unfold any protein domain stabilized by non-covalent interactions (12,13). However, the pili of Gram-positive bacteria are capable of bearing these large perturbations due to the presence of an intramolecular covalent bond, a peptide bond formed between side chain residues. In *S. pyogenes*, the side chains of Lys179 (β–strand1) and Asn303 (β–strand11) of the C-terminal domain of Spy0128 protein form an isopeptide bond, turning this pilin protein into an inextensible molecule (11,14) (Figure S1). Thus, the isopeptide bond becomes the Achilles’ heel of the pilus, since preventing its formation would lead to a mechanical extensible protein, vulnerable to degradation by cellular proteases, hence compromising the bacterial attachment.

In this work, we report the first folding intervention on a bacterial pilus molecule by an external peptide. We targeted the C-terminal domain of the Spy0128 pilin protein with a peptide—*isopeptide-blocker*—which mimics the β-strand11 of the pilin domain. The isopeptide-blocker is expressed prior to the Spy0128 domain, resembling an antibiotic peptide which interferes in the periplasm with a translocated pilin domain before folding. We used a halotagged-peptide to label and purify intervened pilin molecules, and AFM-based force spectroscopy to study the mechanics of the Spy0128 polyprotein. Together, our results indicate that the intervened Spy0128 pilin is completely extensible, shows a low mechanical stability, and is labile to protease digestion due to the compromise of the native
structure. We propose the targeting of the isopeptide bond of Gram-positive pilin molecules, as a rational design of a new generation of peptide antibiotics.

RESULTS AND DISCUSSION

Interfering the intramolecular isopeptide bond

We blocked the formation of the intramolecular isopeptide bond by expressing a competitor peptide which mimics one of the β-strand of the pilin domain. In S. pyogenes, only the C-terminal domain of the Spy0128 experiences forces, thus we targeted it to interfere with the isopeptide bond formed between Lys179 and Asn303 (Figure S1). The isopeptide-blocker is a 19-residues long peptide that mimics the β-strand11 of the C-terminal domain, and contains the Asn residue required to the formation of the isopeptide bond (Figure 1). Hence, the isopeptide-blocker operates as a suicide inhibitor which irreversible binds to Lys179 and block that Asn303 forms the natural isopeptide bond (Figure 1).

We expressed the isopeptide-blocker as a HaloTag fusion protein, which allows, not only a stable expression of the peptide, but also to identify and isolate intervened Spy0128 proteins (Figure 1). Intervened proteins are easily identified by using specific fluorescently labeled halo-ligands in the SDS-PAGE. Moreover, the purified pili protein preparations can be enriched by using HaloTag beads, which bind specifically to the intervened Spy0128 molecules and can be subsequently eluted enzymatically. Figure 2A shows the two constructs used in the protein expression experiments: the 38 kDa halotagged isopeptide-blocker (i), and the 96 kDa polyprotein I27-Spy0128-I27-Spy0128-I27 (ii). Considering the competition between the native β-strand11 and the isopeptide-blocker, a high concentration of the latter is required before the expression of the Spy0128 polyprotein. We selectively expressed each of these proteins using two different inducible expression vectors within the same bacteria (see methods). Only the expression of the isopeptide-blocker followed by the polyprotein yields appreciable amounts of fluorescently decorated Spy0128-polyprotein (Figure 2). The inverted protein expression sequence or the simple induction of only the Spy0128-polyprotein, yields no fluorescently labeled Spy0128 molecule at the molecular weight for single or double decoration (Figure 2).

Protein gels shows that the isopeptide-blocker containing the HaloTag migrates at a molecular weight of 39 kDa, whereas the Spy0128 polyprotein at 98 kDa (using the molecular weight marker as protein standard, see methods) (Figure 2). Thus, the molecular weight of the Spy0128 polyprotein should increases by approximately 40 kDa if a single Spy0128 domain is decorated, or by 80 kDa if both are. The gel in Figure 2C shows a total of five fluorescently labeled bands, where the bands located at 145 and 181 kDa match the predicted molecular weights for single and both Spy0128. The protein band located a low molecular weight (~39 kDa) can be understood as a co-purification of the halotagged isopeptide-blocker, which achieves a high concentration during the protein induction. Nevertheless, the two extra bands located at 67 and 76 kDa do not match any expected molecular weight. We speculate that these bands could arise
from the proteolytic activity of intervened Spy0128 polyproteins, which our proteases inhibitors were not able to prevent during the purification (Figure 2C, right panels). Similar proteolytic susceptibility has been demonstrated in the Glu258Ala Spy0128 protein, a mutant protein where the formation of the isopeptide of the C-terminal domain is abolished (15). In our case, this situation is even more plausible, considering that intervened Spy0128 could be more susceptible to proteolysis due to probable problems with folding triggered by the presence of the isopeptide-blocker.

To validate our isopeptide intervention strategy, we designed two additional peptides to interfere with the structural pili protein of a different organism, Actinomyces oris. In this bacterium, the shaft protein FimA build the pilus, which shows analogous structural features with Spy0128 (16). Although FimA is a pili protein with three β–sandwich domains, only the last two domains operate under force (16). We designed isopeptide blockers which target both the CnaA domain (domain N2) and the CnaB domain (domain N3) (Figure S2). To intervene the domain N2, we mimicked the β–strand1 of the CnaA domain (Isopeptide-N), whereas for the domain N3, we mimicked the β–strandA of the CnaB domain (Isopeptide-K). We followed the same protein expression protocol used for the Spy0128 polyprotein, expressing the isopeptide blocker before the induction of the pili protein. Figure S2 shows that both the isopeptide-N and isopeptide-K blockers successfully decorate the FimA protein, as suggested by the halotagged protein band located at ~40 kDa higher than the undecorated FimA. These experiments confirm that our isopeptide-blocker strategy can be translated to shaft domains from other Gram-positive bacteria.

**Isopeptide blocker prevents the folding of Spy0128**

Through single molecule force spectroscopy, we have previously demonstrated that the presence of the internal isopeptide bond protects Spy0128 from unfolding (14). We characterized the mechanical properties of the intact Spy0128 domain, and mutated Glu258Ala, where the isopeptide bond is abolished(15). In our polyprotein construct, two Spy0128 domains are flanked by I27 titin domains, allowing to use the I27 unfolding pattern as a fingerprint to discard spurious traces (14, 17). While in the intact Spy0128 polyproteins only the I27 domains unfold (Figure 3A), the Glu258Ala mutant is extensible, showing a contour length increment of 50 nm, and an unfolding force of ~300 pN (Figure 3B). These experiments revealed that the internal isopeptide bond prevents the unfolding of the Spy0128 domains.

In contrast, the intervened Spy0128 polyproteins showed typically a long initial extension (L1) —defined as the extension before the unfolding of the first I27 domain (17)—, including several low-stability mechanical intermediates hard to classify (Figure 3C). This suggests that the isopeptide blocker, not only turns Spy0128 into an extensible domain, but also decreases its mechanical stability compared to the mutant Glu258Ala (Figure 3B and C). However, compared to the control, only a fraction of the traces was intervened. We used the L1 as a proxy to estimate the total fraction of protein decorated with the halotagged isopeptide blocker. When the Spy0128 is intact the L1 is 41 ± 12 nm, whereas when one or
both Spy0128 domains are intervened the $L_i$ increases to $80 \pm 16$ nm or $130 \pm 13$ nm, respectively, given the 50 nm contour length increment of each Spy0128 (Figure 3D). From the histogram, we calculated that approximately 20% of the traces are intervened by the halotagged isopeptide blocker (Figure 3D).

Furthermore, the unfolding traces of the intervened Spy0128 show undoubtedly that the mechanical stability is altered (Figure 3C), suggesting that the introduction of an extra $\beta$-strand compromises the entire folding of the Spy0128 C-terminal domain. To discard that this effect is due to the presence of the HaloTag in the isopeptide blocker, we removed the HaloTag through an extra purification step, which also enriched the protein preparation only with intervened Spy0128 molecules. By incubating the polyprotein with Magne-HaloTag-beads (see methods), we washed out the intact Spy0128 and we kept only the intervened Spy0128. We eluted the intervened polyprotein by incubating with TEV, which severs 15 residues far away from the intermolecular isopeptide bond (Figure S1).

Interestingly, AFM experiments using the enriched polyprotein show equivalent unfolding patterns compared to those observed in the undigested TEV polyprotein; extended $L_i$ and low mechanical stability (Figure 3D and Figure 4). Therefore, the presence of the HaloTag is not necessarily related to the low mechanical stability displayed by the intervened Spy0128 polyprotein. Additionally, as a consequence of the enrichment in intervened Spy0128 protein, the $L_i$-based intervened populations are increased up to 60% of the traces (Figure 4, blue and purple population). The large proportion of intact Spy0128 polyproteins (Figure 4, gray population) can be understood by the partial unfolding of intervened Spy0128 showing only two I27 titin domains. However, we recorded several full-length molecules showing the unfolding of three I27 domains, with a $L_i$ of $\sim 40$ nm (Figure 4A, upper panel). We hypothesized that these intact molecules are a contamination during the magnetic separation (Figure 2, Figure 4).

We measured the unfolding force ($F_u$) and contour length increments ($\Delta L_C$) of the multiple mechanical intermediates observed within the traces of the TEV digested Spy0128 polyprotein. These mechanical intermediates cannot be contrasted with the wild-type Spy0128 unfolding, due to its inextensibility. However, the Spy0128 mutant Glu258Ala showed a clear mechanical intermediate (Figure 3B) with $\Delta L_C$ = $50 \pm 1$ nm and $F_u = 293 \pm 64$ pN, although in some occasions the unfolding pathway is through an intermediate with $\Delta L_{C1} = 18 \pm 1$ nm followed by second of $\Delta L_{C2} = 32 \pm 1$ nm(14). These two latter intermediates show less mechanical stability, but cluster together at 182 $\pm$ 41 pN and 111 $\pm$ 15 pN, respectively (Figure 4C). In contrast to the mutant, the isopeptide-blocked Spy0128 polyprotein lacks of defined mechanical intermediates, showing scattered unfolding forces and contour length increments (Figure 4C). These forces are on average under 100 pN, below the stability of the mutant Glu258Ala. From a structural point of view, the mechanical intermediate present in this mutant could be explained by the cooperative rupture of H-bonds between the $\beta$-strand1 and $\beta$-strand11, the mechanical clamp of the molecule (18). The isopeptide blocker mimics the internal $\beta$-strand11, offering the same interactions, including the Asn residue for the formation of the isopeptide bond with Lys179. However, after the intermolecular
bond is established, the natural β–stand1 competes for the same pool of non-covalent interactions, giving rise to steric interactions that would destabilize the fold (Figure S1 and Figure 3C). Our results indicate that the presence of the isopeptide blocker in the mechanical clamp of the molecule interferes with the proper folding of the domain, proved by the lack of stable mechanical intermediates along the unfolding pathway.

CONCLUSION

Our experimental design is based on the intracellular interruption of the intramolecular isopeptide bond and folding of the Spy0128 pilin protein from the Gram-positive bacteria S. pyogenes. Recently, several efforts have been made to manipulate pili proteins from Gram-positive and take advantage of the isopeptide bond (19–21). Howarth and colleagues have implemented an elegant split protein strategy based on several pili proteins (22–24), where the β–strand containing either the Lys or Asn residue is removed and expressed separately form the rest of the protein. Our strategy is inspired by these experiments, however, the isopeptide-blocker system is unique in term that aims to establish a covalent bond without the removal of the natural β–strands. Besides, our results indicate that in addition to successfully forming an isopeptide bond, the isopeptide-blocker interrupts the proper folding of the Spy0128 domain. In contrast to the Spy0128 control, which is inextensible, and to the Glu258Ala non-isopeptide forming mutant which shows a remarkably mechanical stability; the isopeptide-blocked Spy0128 is fully extensible and has a low mechanical stability.

Considering that most the protein folding occurs concomitant with the translation of the polypeptide (25–27), the success of the isopeptide-blocker strategy is based in the timing of the intervention. In our experiments, we express the peptide before the pili protein, increasing the amount of halotagged decorated protein (Figure 2). Therefore, our results suggest that for the success of the intervention the isopeptide-blocker should interfere in the late stages of translation or early of folding. Additionally, taking into account the large amount of protein labeled and digested (truncated), although the presence of inhibitors, we proposed that the isopeptide-blocker interfere with the folding rendering the pili protein susceptible to cellular proteases.

The presence of isopeptide and disulfide bonds in the Gram-positive pili proteins impede that these proteins fold in the cytoplasm, as absence of the bond is required for translocation to the periplasm (28, 29). Pili proteins fold, acquire internal covalent bonds, and polymerize in the periplasm before the formation and anchoring of the pilus to the cell wall (29). In our experiments, as we express the isopeptide blocker in the cytoplasm before the translation of the Spy0128, we are mimicking this physiological condition, where a nascent translocating Spy0128 interacts with the isopeptide blocker within the periplasmic space (Figure 1). We propose this novel strategy to develop a new generation of peptide antibiotics designed to interfere with the mechanical properties of pili molecules, preventing the colonization of tissues.
MATERIALS AND METHODS

Protein engineering and isopeptide-blocker design
We cloned and expressed the structural pili protein from *Streptococcus pyogenes* (Spy0128) (11) and *Actinomyces oris* (FimA) (16). Whereas FimA was expressed as a monomer, which includes both CnaA and CnaB domains; for the Spy0128 protein, we used a previously engineered Spy0128 polyprotein (14). This polyprotein contains two Spy0128 domains separated and flanked by three I27 titin domains, I27-Spy0128-I127-Spy0128-I27. Both constructs, FimA and Spy0128 polyprotein, were cloned in the pQE80L expression vector (Qiagen), which confers ampicillin resistance, and includes a promoter inducible by IPTG and a N-terminal HisTag. The isopeptide-blocker was engineered by concatenating a short peptide sequence from the native β-strand region involved in the isopeptide bond formation from the Cna domain, followed by a TEV site, and the HaloTag sequence. For the Spy0128 we used the peptide –MEFTDKDMTITFTNKKDAE–, which mimics the β-strand11 of the C-terminal CnaB domain (N, asparagine residue that establishes the isopeptide bond). This peptide was followed by –EDIRS– (linker1), –EDLYFQS– (TEV site), –DNTTPE– (linker2), and the HaloTag.

In the case of the FimA, considering that both domains experience force during the bacterial attachment (16), we targeted the CnaA (N2 domain) as the CnaB domain (N3 domain). For the domain N2 we used –MEFARNGAITNRAQVSD–, which mimics the β-strand11, penultimate β-strand of the CnaA domain, whereas for the domain N3 we used –MEFWGDLIIKKVDNHQOG–, which mimics the β-strandA, first β-strand of the CnaB domain. Both peptides were followed by –HGVRS– (linker1), TEV site, –DNTTPE– (linker2), and the HaloTag. The three isopeptide-blocker constructs were cloned in a custom modified pBAD18 expression vector (ATCC), which confers Kanamycin resistance, it is inducible by arabinose and lacks of a HisTag.

Protein expression, purification, and labeling
The co-expression of the pili protein and the halotagged isopeptide-blocker was conducted in two stages. First, pQE80L and pBAD co-transformed *E. coli* BLR pLysS cells were grown at 37° C in M9CA broth (VWR) supplemented with 40 μg·mL⁻¹ thymine, 2 mM magnesium sulfate, 0.1 mM calcium chloride, and 1.5 % glycerol as carbon source. When the culture reached an OD₆₀₀ of 0.5-0.6, the expression of the isopeptide-blocker was induced with 0.2% of arabinose. After growing the cells at 37° C for three hours, the culture was centrifuged, suspended in LB broth, and incubated for 15 min at 37° C. Second, the cells were grown for three additional hours at 37° C in LB broth supplemented with a final concentration of 1 mM IPTG, for the expression of the pili protein. Finally, the cells were harvested by centrifugation, washed in phosphate buffer and stored at -80° C. In the control where the Spy0128 polyprotein was induced before the isopeptide-blocker, we inverted protocol growing first the bacteria in LB broth, followed by the minimum media.

The pili proteins were purified using the protocol described previously (14) (30). Briefly, after that the *E. coli* cells were lysed by French press in phosphate buffer
(50 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 300 mM NaCl), the proteins were purified by Ni-affinity chromatography (QIAGEN), followed by a step of size exclusion (Superdex S200, GE healthcare) in Hepes buffer (10 mM Hepes buffer pH 7.2, 150 mM NaCl, 1 mM EDTA). Protein fractions after the Ni-affinity and size exclusion chromatography were labeled with 1 mM of the fluorescent dye Alexa488-Halo-ligand (Promega) and analyzed by SDS-PAGE. The protein gels were imaged in a documentation gel station (G-box, Syngene) using epi illumination and a proper filter set. The molecular weight of the intact pilin proteins, isopeptide-blockers, and the decorated FimA and Spy0128 polyprotein, was calculated through Gel-Pro Analyzer 3.1 (Media Cybernetics) using the molecular weight standard as reference (precision plus protein dual color, Bio-Rad).

Selected Spy0128 polyprotein fractions were concentrated to a volume of ~50 μL and incubated with 50 μL of pre-equilibrated Magne-HaloTag-beads (Promega) for >4 hours at 4°C in a tube rotator. The intact polyproteins were washed 3 times in Hepes buffer by centrifugation for 3 min at 1000 g at 4°C. The covalently bonded intervened proteins were eluted by incubation of the beads with 100 units of ProTEV Plus (Promega) at 4°C in Hepes buffer overnight in the tube rotator. Finally, we applied a magnet to separate the proteins from the Magne-beads. TEV treated fractions were stored at 4°C until further experimentation.

**Atomic Force Microscope Experiments**

Polyproteins purified with or without the extra Magne-HaloTag-bead TEV step were incubated for 30 minutes in freshly nickel-chromium-gold evaporated glass cover slides. The experiments were conducted at room temperature using a custom built AFM or the commercial available atomic force spectrometer (Luigs and Neumann). Each of the silicon nitride cantilevers (MCLT) used in the pulling experiments was calibrated following the equipartition theorem (31), giving a spring constant of 10-20 pN·nm⁻¹. The polyproteins were picked by pushing 1-2 nN and stretching single molecules with a pulling velocity of 400 nm·s⁻¹. All the experiments were done in Hepes buffer at room temperature.

**Single Molecule Data Analysis**

We used the criteria established for chimeric protein constructions: only traces containing two or three I27 unfolding events were considered for the analysis (14, 17). As the I27 domains are flanking the Spy0128, the traces included within the results should contain the unfolding of the pilin domain. The initial (Lᵢ) and the final extension (Lᵢ) of the polyprotein, the contour length increments of I27 (ΔLᵢ C I27), and the pilin intermediates (ΔLᵢ C Spy) were analyzed using the elasticity polymer model worm-like chain (32). Histograms were fitted using single and multiple Gaussian distributions implemented in Igor 7 (WaveMetrics).

**Author contributions**

J.A.R.P. and J.M.F. designed the research project; J.A.R.P. and C.L.B. did the protein expressions; J.A.R.P. conducted the protein labeling and TEV digestion experiments; J.A.R.P. and R.T.R. conducted and analyzed the single molecule experiments; J.A.R.P., R.T.R., and J.M.F. wrote the article.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References


Figure 1. Strategy for the intervention of the Spy0128 C-terminal domain by the isopeptide blocker peptide. (A) Bacteria use multiple pili to adhere to their substrates. In the Gram-positive bacteria *S. pyogenes*, the pilus is formed by three pilin proteins: Spy0130, or base of the pilus (*pink*); Spy0128, which forms the rod (*gray and yellow*); and the Spy0125, which contains the binding site for the surface ligand (*green*). Mechanical forces are transmitted along the pilus, but the presence of isopeptide bonds within the structure of the pilin Spy128 prevent mechanical unfolding (Figure S1). The intervention of the isopeptide bond in the C-terminal domain can compromise the stability of Spy0128, triggering unfolding under mechanical stresses (white arrows). (B) The intramolecular isopeptide bond is formed between the Lys179 and Asn303 located in the β-strand 1 and 11, respectively. However, the molecule should be fully translated before the formation of the bond (i). The engineered halotagged isopeptide blocker is expressed before the Spy0128 polyprotein facilitating the encounter with the nascent pilin. (ii) The halotagged isopeptide-blocker forms an isopeptide bond with the freshly synthetized Spy0128 C-terminal domain, abolishing the native bond. (iii) To confirm the intervention of the pilin domain, the protein is incubated with Alexa488-Halo-ligand and analyzed by SDS-PAGE. (iv) To control that the HaloTag has not effect on the folding state of Spy0128, the protein construct is incubated with paramagnetic Halo-beads and eluted by adding TEV enzyme to the solution (see methods).
Figure 2. The isopeptide blocker forms a covalent intermolecular bond with the pilin Spy0128. (A) Protein constructs used for the intervention of the intramolecular isopeptide bond. (i) The isopeptide blocker consists of three segments: the β–strand11 of the Spy0128 C-terminal domain, a flexible linker which contains the TEV site, and finally the HaloTag enzyme. (ii) The Spy0128 polyprotein construct, consisting of two Spy0128 domains flanked by three I27 domains used as the mechanical fingerprint. (B) SDS-PAGE of Ni-NTA purified proteins showing the differential expression of the isopeptide-blocker and Spy0128 polyprotein labeled with Alexa488 halo-ligand (left) and coomassie blue for total protein staining (right). (IG= IPTG induction, IGA= IPTG followed by arabinose induction, IAG= arabinose followed by IPTG induction). Only the induction of the isopeptide blocker followed by Spy0128 polyprotein yields a detectable amount of labeled polyprotein. (C) SDS-PAGE of Spy0128 protein fractions purified by Ni-NTA resin. Because the halotagged isopeptide blocker lacks of HisTag, the peptide is washed out after the incubation with the resin, which retains only the intact and intervened Spy0128 polyproteins. The molecular weight of the fluorescently labeled protein calculated is: 181, 145, 76, 67, and 39 kDa (estimated using the MW as reference). (Right), Cartoon showing the interpretation of the bands found in the fluorescent gels. Green star corresponds to the Alexa488 halo-ligand molecule.
Figure 3. Halotagged isopeptide blocker interferes with the folding of the C-terminal domain of Spy0128. (A-C) Mechanical fingerprint of the isopeptide bond. (A, left) C-terminal domain of the Spy0128 protein. (Middle) The intramolecular isopeptide bond connects the side chains of Lys179 and Ans303, limiting the extension of the protein. (Right) Force-extension trace showing the mechanical unfolding of the three I27 domains—which occurs at ~200 pN—as the intact Spy0128 is mechanically inextensible. (B, left and middle) Mutant Spy0128 where
the catalytic Glu258 is substituted to Ala, abolishing the formation of the intramolecular isopeptide bond. (Right) In this case, the force-extension recording shows the unfolding of the three I27 domains followed by the unfolding of the two C-terminal Spy0128 domains. I27 domains unfold at 200 pN, whereas C-terminal Spy0128 unfolds at >300 pN. The $L_i$ is unaltered compared with the wild-type Spy0128, but the $L_f$ is increased due to the unfolding of the Spy0128 C-terminal domain. (C, left and middle) Spy0128 forming the isopeptide bond between the Lys171 and the Asn residues present in the isopeptide blocker. (Right) Here, the trace shows the unfolding of the three I27 domains, preceded by several low force mechanical intermediates, fingerprint of the intervened Spy0128. In this case, the $L_i$ is similar to the extensible mutant Glu258Ala; however, the $L_f$ increases notably due to the contribution of the intervened Spy0128. Arrowheads indicate the direction of the cantilever force-position (gray = approaching, black = retraction). (D) Initial extensions ($L_i$) of the halotagged intervened Spy0128. The $L_i$ is the extension of the polypeptide before the unfolding of the I27 domains (see text), calculated by the WLC. Three populations are detected in the accumulative histogram: intact domains with an extension of 41 ± 12 nm (gray population), single intervened Spy0128 domain with 80 ± 16 nm (blue population), and finally the double intervened with 130 ± 13 nm (purple population). Inset, contour length increments ($\Delta L_c$) and unfolding force ($F_u$) of halotagged intervened Spy0128 polyproteins.
Figure 4. The Isopeptide blocker increases the extensibility and decreases the mechanical stability of Spy0128 domain. (A) Typical unfolding traces found in the TEV digested (HaloTag removed) intervened Spy0128 polyprotein. Three different kinds of molecules were found: (i) intact Spy0128 proteins, where only I27 are detected; (ii) polyproteins with one intervened Spy0128 domain, which is extensible but low mechanical stability, while the other spy0128 domain remains inextensible; (iii) polyproteins where both Spy0128 are intervened, lacking of mechanical stability, and showing several weak intermediates. (B) Initial extension ($L_i$) of the Magne-Halo beads TEV eluted proteins. Three peaks are detected in the accumulative histogram: (i) intact domains, with an extension of $34 \pm 15$ nm (gray population); single intervened Spy0128 domain, $L_i = 86 \pm 16$ nm (blue population); and double intervened, $L_i = 135 \pm 21$ nm (purple population). The results are consistent with the $L_i$ found in the intervened halotagged Spy0128 polyproteins (Figure 3). (C) Two-dimensional histogram showing the contour length increment ($\Delta L_C$) and unfolding force ($F_U$) of the mutant Glu258Ala (left) and the intervened Spy0128 (right). The mechanical unfolding of the mutant domain occurs through two different mechanical pathways: $80\%$ of the times it unfolds in one single event with $\Delta L_C = 50 \pm 1$ nm and $F_U = 293 \pm 64$ pN, while $20\%$ of the times through two mechanical intermediates, $\Delta L_C^1=18 \pm 1$ nm and $182 \pm 41$ pN, followed by $\Delta L_C^2 = 32 \pm 1$ nm with a $F_U$ of $111 \pm 15$ pN, respectively(14). The presence of the isopeptide blocker compromises significantly the mechanical stability of the pili domain, which unfolds through several non-identified intermediates below 100 pN. Scale color bars indicate the total number of events.