Single-molecule Force Spectroscopy Reveals the Individual Mechanical Unfolding Pathways of a Surface Layer Protein*

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Surface layers (S-layers) represent an almost universal feature of archaeal cell envelopes and are probably the most abundant bacterial cell proteins. S-layers are monomolecular crystalline structures of single protein or glycoprotein monomers that completely cover the cell surface during all stages of the cell growth cycle, thereby performing their intrinsic function under a constant intra- and intermolecular mechanical stress. In Gram-positive bacteria, the individual S-layer proteins are anchored by a specific binding mechanism to polysaccharides (secondary cell wall polymers) that are linked to the underlying peptidoglycan layer. In this work, atomic force microscopy-based single-molecule force spectroscopy and a polyprotein approach are used to study the individual mechanical unfolding pathways of an S-layer protein. We uncover complex unfolding pathways involving the consecutive unfolding of structural intermediates, where a mechanical stability of 87 pN is revealed. Different initial extensibilities allow the hypothesis that S-layer proteins adapt highly stable, mechanically resilient conformations that are not extensible under the presence of a pulling force. Interestingly, a change of the unfolding pathway is observed when individual S-layer proteins interact with secondary cell wall polymers, which is a direct signature of a conformational change induced by the ligand. Moreover, the mechanical stability increases up to 110 pN. This work demonstrates that single-molecule force spectroscopy offers a powerful tool to detect subtle changes in the structure of an individual protein upon binding of a ligand and constitutes the first conformational study of surface layer proteins at the single-molecule level.

Crystalline bacterial cell surface layers, referred to as S-layers (1), have now been identified in hundreds of different species from nearly every taxonomical group of walled bacteria and represent an almost universal feature in archaea (2). S-layers, which are formed by a highly specific and robust self-assembly process of S-layer proteins, constitute the simplest type of protein membrane developed during evolution. S-layer proteins self-assemble into monomolecular lattices with different symmetries ranging from p1 to p6, thereby exhibiting pores of identical size and morphology. As these crystalline layers represent the outermost barrier between the living cell and its environment, they are supposed to play a crucial role regarding stabilization and protection of the cell (3), which implies a certain resistance against physiologically relevant forces (4–6). The remarkable feature of S-layer proteins to self-assemble also in vitro has aroused the interest of nanotechnologists to use them as a matrix for molecular construction kits following a bottom-up approach (7). Although considerable knowledge has been accumulated on the genetics, the self-assembly behavior, the structure-function relationship, and the biochemical as well as biophysical properties of S-layer proteins (7–18) an overall tertiary structure of one native unmodified protein remains elusive. Classical experimental structure determination methods such as NMR and x-ray crystallography pose problems because of the size and two-dimensional crystallization behavior of S-layer proteins, as in solution they form crystalline monomolecular layers rather than isotropic three-dimensional crystals. The dissolved proteins immediately interact to form small oligomers, which provide the nucleation seed for the formation of large layers (19). Recently, a structural model of an S-layer protein, namely the protein SbsB of Geobacillus stearothermophilus pV72/p2 (20) could be determined by means of molecular dynamics simulations and small-angle x-ray scattering (21, 22). This S-layer protein is made up of 920 amino acids, has a molecular weight of 98 kDa, and forms two-dimensional layers exhibiting p1 lattice symmetry. SbsB possesses three N-terminal S-layer homologous (SLH) domains (23), which are of α-helical character, and fibronectin type III as well as Ig-like domains, which are composed of β-sheets. The SLH domains are located at the N terminus of the protein within the first 208 amino acids (24). This structural architecture is very common for proteins located on the surfaces of cells, which have been successfully investigated using atomic force microscopy-based single-molecule force spectroscopy (25–27). The SLH domains enable the binding of non-classical secondary cell wall polymers (SCWPs) to anchor the individual proteins to the underlying cell wall (24, 28–30). The SCWP of G. stearothermophilus pV72/p2 was shown to consist of substoichiometrically substituted tetrasaccharide repeats of the following structure: \((\beta-D-Glc\beta\text{NAC})_{0.3-4}\)-\((\beta-D-Man\text{NAC})_{1-3}\)-\((\beta-D-Glc\beta\text{NAC})_{0.3-4}\).
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Atomic force microscopy-based single-molecule force spectroscopy offers an opportunity to study the mechanical unfolding pathways of individual proteins, thereby elucidating the mechanical stability and possible transition states (25, 34–41). S-layer proteins perform their intrinsic function under constant mechanical stress because of the fact that they completely cover whole cells during all stages of the cell growth and cell division (3, 42). To better understand the structure-function relationship and the role SCWPs play during the transport of S-layer proteins to the cell surface, it is of great importance to study the mechanical unfolding of individual proteins under the influence of a pulling force. Single-molecule force spectroscopy was already successfully used to investigate the influence of a ligand on the mechanical unfolding of various proteins. It was reported that because of ligand binding the mechanical stability of proteins often increases even when the binding event takes place at a position far from those regions that are critical for mechanical stability. However, the mechanical unfolding pathway remains the same (43–49). On the contrary, mechanosensitive proteins show a conformational adaption to mechanical stress. Mechanical force can induce a conformational adaption to the mechanical unfolding pathway remains the same (43–49). On the contrary, mechanosensitive proteins show a conformational adaption to the mechanical unfolding pathway remains the same (43–49). On the contrary, mechanosensitive proteins show a conformational adaption to mechanical stress. Mechanical force can induce a conformational adaption to mechanical stress. Mechanical force can induce a conformational adaption to mechanical stress. Mechanical force can induce a conformational adaption to mechanical stress. 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A

B

C

D

E

F

G

H

12.1 ± 4.9 nm

86.8 ± 12.5 pN

36.3 ± 5.2 nm

87.6 ± 10.8 pN

28.14 ± 2.2 nm

197.89 ± 16.2 pN
fied pET3 vector were digested with BssHII (Invitrogen) and then ligated using T4 DNA ligase (Invitrogen), yielding the vector pSbsB-I27. In the plasmid, the coding sequence of the S-layer protein SbsB is flanked by two and three coding sequences of I27 domains, respectively (Fig. 1A). Finally, the plasmid was transformed into E. coli BL21 cells by electroporation. Protein expression was performed as described previously (58). The polyproteins were purified by nickel–His affinity chromatography using 1-ml HisTrap™ HP prepacked columns (GE Healthcare) and anion exchange chromatography using Uno Q-1 columns (Bio-Rad), both under non-denaturing conditions. Finally the proteins were dialyzed against MilliQ water containing 1 mM DTT, yielding a protein concentration of 0.13 mg/ml. The purity of the protein was verified by SDS-PAGE.

**Purification and Modification of SCWP**—The SCWP of *G. stearothermophilus* pV72/p2 was isolated and pyridyl disulfide-activated as described previously (28, 59).

**Single-molecule Force Spectroscopy**—Forforce-extension measurements were conducted using the JPK NanoWizard I atomic force microscope. To study the mechanical unfolding of the polyprotein, 20 μl of the protein solution (0.13 mg/ml) was deposited on a freshly evaporated gold coverslide, incubated for 30 min, and rinsed thoroughly with MilliQ water. For the investigation of the influence of the ligand, 20 μl of SCWP solution (1 mg/ml) was deposited on a freshly evaporated gold coverslide, incubated for 30 min, and rinsed with MilliQ water. Subsequently, 30 μl of the protein solution was added and incubated for 60 min. The measurements were performed using MSCT cantilevers (Bruker, AXS), which were individually calibrated using the equipartition theorem (60), resulting in a typical force constant of 16 pN/nm. Individual proteins were picked up from the surface by gently touching the coverslide with the atomic force microscopy cantilever tip to promote the nonspecific adhesion of the proteins to the cantilever and extended by retracting the cantilever with a constant pulling velocity of 400 nm/s. The resulting force-extension traces were fitted to the worm-like chain (WLC) model of polymer elasticity (61) using a Mathematica script. We used a constant persistence length of 0.4 nm (40).

**RESULTS**

**Stepwise Mechanical Unfolding of the S-layer Protein SbsB**—Fig. 2, A and B, shows two typical force-extension curves corresponding to the unfolding of (I27)2-SbsB-(I27)3. We observe five consecutive unfolding events corresponding to the full extension of the five I27 domains at ~200 pN with a contour length increment of ~28 nm. The distribution of forces and increments in contour lengths for the mechanical unfolding of I27 are shown in Fig. 2, G and H, which is in good agreement with published values (55) and can, therefore, be exclusively related to the mechanical unfolding of I27. Prior to the unfolding of the double peak is \( \Delta L_1 = 12.1 \pm 4.9 \text{ nm} \) (C) and between the second peak of the double peak and the first I27 peak \( \Delta L_2 = 36.3 \pm 5.2 \text{ nm} \) (F). The mean unfolding force of the first peak is \( 86.8 \pm 12.5 \text{ pN} \) (D) and \( 87.6 \pm 10.8 \text{ pN} \) (F), respectively (n = 90). G and H, histograms of the contour length increase and the unfolding forces of the I27 domains. The mean contour length increment is 28.14 ± 2.2 nm, and the mean unfolding force 197.89 ± 16.2 pN for n = 400.
observed. In the case of polyQ chains, the absence of a complete protein extension was explained by the formation of compact and highly stable conformations that are mechanically resilient. Moreover, a connection between this ensemble of mechanically resilient conformations and the assembly process of the proteins was postulated. The lack of a complete initial extension of S-layer proteins might be similarly explained through the existence of compact and highly stable conformations of S-layer proteins that are extensile in the framework of the experiment.

Increase of the Mechanical Stability and an Apparent Conformational Change of SbsB When Bound to SCWP—In Fig. 1B, the setup for this experiment is shown schematically. SCWP is responsible for anchoring the monomers via the N-terminal SLH domains to the rigid cell wall. The exact amino acids comprising the three SLH domains of SbsB are not yet known, but the overall location at the N-terminal region could be revealed by various experimental methods (24, 28) and is shown in a magnified view in Fig. 1B. This interaction is specific for G. stearothermophilus pV72/p2 and ensures that the crystalline protein layer is constrained to the cell surface. Fig. 4, A and B show two typical force-extension traces corresponding to the unfolding of (I27)2-SbsB-(I27)3 in the presence of SCWP molecules. Again, the five consecutive peaks resulting from the unfolding of five I27 domains can be detected. Prior to the unfolding events related to the I27 domains, we again observe a double peak as for the protein monomers. Interestingly, an additional unfolding event appears in the presence of the ligand, which is marked with a green asterisk in Fig. 4, A and B. This unfolding event takes place posterior to the double peak and marks an additional intermediate structure of SbsB that has not been observed for the unbound monomers. The increase in contour length between the first and second peak of the double peak is slightly larger than for the protein without SCWP, ΔL1 = 17.3 ± 4.2 nm (Fig. 4C, ΔL1). The contour length increment between the double peak and the consecutive new peak equals 44.9 ± 6.5 nm (Fig. 4E, ΔL2) and the increase in contour length before the first I27 domain unfolds equals 32.8 ± 7.2 nm (Fig. 4G, ΔL3). The force necessary to unfold the consecutive unfolding intermediates is about 110 pN for the two peaks of the double peak, respectively, and about 117 pN for the new peak and, consequently, about 30% higher than for the unbound protein (Fig. 4, D, F, and H show the histograms for n = 64). Hence, the binding of SbsB to its specific secondary cell wall polymer results in an increase in mechanical stability and to a change of the mechanical unfolding pathway revealed by an additional unfolding peak.

DISCUSSION

The polyprotein approach used in this work allows the individual study of the conformations of an S-layer protein that, when placed under solution conditions, exhibits irreversible two-dimensional crystallization. Our experiments at the single-molecule level demonstrate that the S-layer protein SbsB is not fully extensible under the presence of a pulling force. Apparently, the complete protein cannot be mechanically extended. By combining polyprotein engineering and single-molecule force spectroscopy, we can be certain that individual S-layer proteins are being mechanically unfolded. This confidence results from obtaining an unambiguous molecular fingerprint established by the accurate unfolding of flanking I27 domains. A double-peak of about 87 pN that occurs prior to the mechanical unfolding events related to the I27 domains serves as a mechanical fingerprint for the individual unfolding of certain domains of SbsB, revealing a rather complex unfolding pathway through distinct, well defined intermediates where different initial extensibilities are observed. Such a phenomenon has been observed previously for the mechanical unfolding of polyQ chains (63), the human tau protein (64), and α-synuclein (65), where these proteins are all linked to the formation of self-assembled aggregates involved in the pathology of neurodegenerative diseases. On the basis of single-molecule force spectroscopy measurements, the aggregation propensity of these proteins could be related to certain conformational properties, where an ensemble of mechanically resilient, highly stable collapsed states could be observed. This formation of complex, disordered structures might be in all cases a signature of the onset of protein aggregation. Interestingly, S-layer proteins, which also exhibit the intrinsic property to self-assemble into two-dimensional structures in solution, show the same mechanical unfolding pattern. Therefore, the lack of a complete initial unfolding allows the hypothesis that self-assembling or self-associating proteins might adapt an ensemble of highly stable and mechanically resilient collapsed states which, in the case of S-layer proteins, promote their intrinsic feature to self-assemble into ordered functional structures.

After a variable initial extension, the mechanical unfolding pattern of SbsB shows a clear double peak related to at least two structurally closely located domains. Similar unfolding patterns have been reported for fibronectin type III modules (25), OspA (66), kinase domains (67), T4 lysozyme (68), and the maltose-binding protein (34). SbsB is expected to consist of fibronectin type III and Ig-like domains, structures of known mechanical stability, which allows for the assumption that the double peak originates from the unfolding of such domains in the protein. Thermally and chemically induced unfolding studies also indicated that SbsB is a multidomain protein exhibiting a complex unfolding behavior, with a three-step unfolding having been observed (16). A contour length increase of about 12 nm indicates that the consecutive unfolding intermediates are closely located in the structure of the protein. Because of the lack of a Protein Data Bank file for the atomistic structure of this S-layer protein, the transition states observed in the unfolding studies

FIGURE 4. Mechanical unfolding of the S-layer protein SbsB when bound to its specific SCWP. A and B, typical force-extension traces. The five consecutive peaks (WLC fit in orange) at about 200 pN correspond to the unfolding of the five I27 domains. The three peaks prior to the I27 peaks correspond to SbsB (blue, red, and green WLC fit). The additional peak that can be observed is marked with a green asterisk. The contour length increase between the first and the second peak of the double peak is ΔL1 = 17.3 ± 4.2 nm (C), between the second peak of the double peak and the third peak ΔL2 = 44.9 ± 6.5 nm (E), and between the third peak and the first I27 peak ΔL3 = 32.8 ± 7.2 nm (G). The unfolding forces are higher than for the unbound protein, namely F1 = 110.1 ± 9.1 pN (D), F2 = 109.9 ± 9.8 pN (F), and F3 = 117.4 ± 9.7 pN (H).
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cannot be assigned to certain structural conformations. However, comparable forces have been found for fibronectin (27, 69) and tenascin (26), both extracellular matrix proteins, and again for a fibronectin type III domain (25). Hence, a mechanical stability of about 87 pN and the existence of a double peak prove that this S-layer protein also exhibits mechanically stable conformations whose extension implies the overcoming of measurable unfolding energy barriers.

The constant maintenance of a coherent S-layer lattice is important for acting as a barrier and well defined molecular sieve (3, 70). The unfolding pattern of individual S-layer proteins indicates a significant mechanical stability of S-layers, which plays an important role for their function as highly dynamic closed surface crystals (42, 71, 72).

S-layers are anchored to the cell wall through the binding of species-specific SCWPs, where the binding takes place at the N-terminal region of the protein involving three SLH domains (73). When individual proteins are mechanically unfolded in the presence of these ligands, an additional unfolding peak appears posterior to the double peak that was observed for the unbound proteins. Apparently, ligand binding triggers a conformational change in the protein that stabilizes some key interactions that, once placed under force, result in an extra force peak in the unfolding traces upon disruption. Because of the lack of structural information of SbsB at an atomistic level, we are not able to relate the additional transition state to a specific protein conformation. However, the altered pathway upon ligand binding demonstrates that such conformational changes can be sampled using single-molecule force spectroscopy. S-layer proteins apparently adopt a different conformation when bound to their specific ligands. It has been found recently that S-layer proteins adopt different conformations as monomers and when part of self-assembled structures (8, 74). Obviously, such a conformational change also takes place when S-layer proteins bind to SCWPs. Recent studies on the mechanoenzymatics of titin kinase showed that mechanical force is able to induce a catalytically competent conformation to bind substrates (50), where an additional peak was observed caused by the interaction with the ligand (51). Similar studies on mechanosensitive proteins demonstrate that because of the extension and stretching of a protein, active intermediate conformations become accessible for the binding of specific substrates (41, 52, 53, 75, 76). In our experiments, S-layer proteins bind SCWPs prior to any mechanically induced unfolding event. Thus, the observed conformational change takes place because of the binding of the ligand rather than because of an extension of the protein. The additional force peak provides an extra constraint to the protein to mechanically unfold, which gives new insights into the behavior of S-layer proteins in vivo.

The morphogenesis of S-layers involves the translocation of monomers through the peptidoglycan-containing rigid cell wall matrix, the binding to SCWPs, and the incorporation in the growing closed S-layer lattice in a defined orientation. The exact secretory pathway of S-layer proteins is not yet well understood. However, the high molecular mass and the complex tertiary structure of S-layer proteins lead one to assume that monomers are transported through the cell wall in a rather unfolded conformation. The observed conformational change of an S-layer protein together with experimental findings related to the behavior of S-layers within the peptidoglycan layer (33) provide a possible explanation of how the complex translocation of individual proteins through the cell wall might be controlled. S-layer monomers constitute an S-layer protein pool “waiting” in the peptidoglycan matrix for the upcoming insertion into the S-layer on the surface of the growing and dividing cell. The binding of SCWPs might induce a crucial conformational change that stabilizes certain key interactions, enabling the interaction with other S-layer proteins, where this intermolecular interaction leads to a final conformational change yielding the native structure within an S-layer.

Various single-molecule experiments have been conducted to investigate the influence of a bound ligand on the mechanical stability of different proteins (43–48). Independent of the binding site, it was observed that the mechanical stability of the protein increases in most of the cases when the ligand is bound. Here we monitor the same phenomenon. The mechanical stability increases from about 87 pN up to about 110 pN, which represents a significant increase of about 30%. The additional unfolding event marks an even higher mechanical resistance of about 117 pN. This enhanced mechanical stability arises despite the fact that the cell wall polymers do not directly interact with the key region that is supposed to be important for mechanical stability. The SCWPs bind to the N-terminal region of the protein, which is mainly composed of α-helices (21). α-helical structures are reported to unfold under the presence of much lower forces than observed in our experiments (34, 77–79). Therefore, the mechanical stability is likely to arise because of β-sheet structures, which are located closer to the C-terminal region where the protein consists of Ig-like and fibronectin type III domains (21). Apparently, a long-range coupling, as reported previously (45), can be also observed for S-layer proteins.

Our experiments provide the first demonstration of a change of the mechanical unfolding pathway because of the binding of a specific ligand prior to any force-induced extension of the protein, which serves as a direct indication of a conformational change of the protein. Our results clearly demonstrate the mechanical unfolding of an S-layer protein through distinct, well defined intermediates, where the lack of a complete initial extension of the protein indicates the formation of an ensemble of mechanically resilient collapsed states; a phenomenon that was observed previously for aggregating proteins. These results identify S-layers as mechanically highly stable protein layers able to bear the mechanical stress that prokaryotic cells permanently experience in nature.

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