On the remarkable mechanostability of scaffoldins and the mechanical clamp motif

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Protein mechanostability is a fundamental biological property that can only be measured by single-molecule manipulation techniques. Such studies have unveiled a variety of highly mechanostable modules (mainly of the Ig-like, β -sandwich type) in modular proteins subjected to mechanical stress from the cytoskeleton and the metazoan cell–cell interface. Their mechanostability is often attributed to a “mechanical clamp” of secondary structure (a patch of backbone hydrogen bonds) fastening their ends. Here we investigate the nanomechanics of scaffoldins, an important family of scaffolding proteins that assembles a variety of cellulases into the so-called cellulosome, a microbial extracellular nanomachine for cellulose adhesion and degradation. These proteins anchor the microbial cell to cellulose substrates, which makes their connecting region likely to be subjected to mechanical stress. By using single-molecule force spectroscopy based on atomic force microscopy, polypeptide engineering, and computer simulations, here we show that the cohesin I modules from the connecting region of cellulosome scaffoldins are the most robust mechanical proteins studied experimentally or predicted from the entire Protein Data Bank. The mechanostability of the cohesin modules studied correlates well with their mechanical kinetic stability but not with their thermal stability, and it is well predicted by computer simulations, even coarse-grained. This extraordinary mechanical stability is attributed to 2 mechanical clamps in tandem. Our findings provide the current upper limit of protein mechanostability and establish shear mechanical clamps as a general structural/functional motif widespread in proteins putatively subjected to mechanical stress. These data have important implications for the scaffoldin physiology and for protein design in biotechnology and nanotechnology.

cellulosome | cohesin | mechanical stability | protein nanomechanics | single-molecule force spectroscopy

Mechanical forces are ubiquitous in biological systems. However, we have only recently learned how to experimentally control and measure this new biophysical parameter through single-molecule manipulation. Such studies have revealed that many proteins generate, sense, transmit, or are somehow subjected to mechanical forces during their normal functioning. A subset of these “mechanical proteins” is responsible for maintaining structural integrity in vivo and they display widely different mechanical stabilities, measured in vitro as the most probable unfolding force, F_u (after having been pulled apart, normally in the N–C direction). Such forces range from the pure entropic elasticity regime (e.g., elastomers like elastin) to a few tens of piconewtons (pN) (e.g., spectrin) and to slightly above 300 pN (titin immunoglobulin-Ig-domains) (1–5).

Mechanical proteins tend to be multimodular and the modules they are made of are often of the same type, although they commonly display distinct mechanical stabilities. These mechanical modules frequently seem to act as shock absorbers that protect critical biological interactions from mechanical stress, particularly in the metazoan cell–cell interface (3–5). Their

mechanostability is often attributed to a “mechanical clamp” of secondary structure that fastens their ends and, to date, Ig modules appear to be among the most mechanically stable ones.

Here we have investigated the nanomechanics of scaffoldins (Fig. 1), a family of microbial modular proteins that are particularly motivating from the mechanical point of view for 3 reasons: (i) Scaffoldins are noncatalytic structural proteins of the cellulosome, a multienzyme, cell-surface complex required for adhesion and degradation of crystalline cellulose, a particularly recalcitrant substrate (Fig. 1A). These scaffolding proteins act as a molecular Lego, binding a number of cellulases through its type I cohesin (cohesin I) modules to spatiotemporally regulate the efficiency of the entire enzymatic cascade (6). Furthermore, they physically anchor the microbial cell (6, 7) to the crystalline cellulose substrate (8), although the quaternary structure of the model cellulosomes, including their linkages to the cell and substrate, is highly polymorphic and not yet well resolved (Fig. 1A; ref. 6). The bonds joining the scaffoldin system are very tenacious, which include several of the highest affinity, noncovalent bonds known in nature [interestingly, one such bond in some scaffoldins is even covalent (9)], suggesting that they may be part of a stable mechanical circuit (Fig. 1A). Like in cell adhesion proteins from metazoans (5), we hypothesize that under physiological conditions, the scaffoldin region located between the 2 key attachment points (cell and substrate) might be exposed to a greater axial mechanical stress. We call this the “connecting region,” which is a segment putatively subjected to a more intense mechanical stress than the rest of the protein, referred to as the “hanging region”. (ii) The cohesin I modules are jellyroll domains with an Ig-like, β -sandwich topology. A simple inspection of their structure reveals a mechanical topology similar to that of titin Ig domains (Fig. 1B). However, the presence of longer putative mechanical clamps of β -sheet secondary structure in cohesins suggests that they may have a higher mechanical stability. (iii) Finally, it was suggested that a linear hydrodynamic flow could mechanically disrupt the integrity of cellulosomes releasing the bound cellulases (10), thus providing indirect evidence that the mechanical integrity of this extracellular complex is important for its function.

Results and Discussion

We have studied 2 well-characterized model scaffoldins from motile Clostridia: CipA from *Clostridium thermocellum* (a ther-

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Fig. 1. Quaternary structure of scaffoldins and the atomic structures of the cohesin I modules studied. (A) Simplified cartoon representation of the current models for the architecture of 2 widely used model systems of closely related scaffoldins: CipA from *Clostridium thermocellum* (Top) and CipC from *C. cellulolyticum* (Bottom). Scaffoldins, the cellulase-integrating scaffolds of the cellulosome, are linked to the bacterium through anchoring proteins via their dockerin II modules, and to the substrate through a family-3, cellulose-specific, carbohydrate-binding module. The connecting region (contained in the 2 constructs analyzed in Figs. S1 and S2) is located between the 2 anchoring points and it is indicated by a black line. The 3 scaffoldin modules analyzed in this study are of the cohesin I type and they are indicated by colored asterisks: c7A, red; c2A, orange; and c1C, pink (this color code will be followed throughout this article). These modules were selected because their atomic structures were available, a prerequisite for MD studies. According to our working hypothesis, modules c7A and c1C (belonging to the connecting region) would be subjected to a greater mechanical stress than c2A (from the hanging region). (B) Mechanical topology of cohesin I modules. Atomic structures of c7A cohesin I module (Right) from *C. thermocellum* and, of the I27 module from human cardiac titin, a protein from the sarcomeric cytoskeleton, which is a standard in protein nanomechanics, used here for comparison (Left). The I27 module is a 7-stranded β -sandwich lig-fold with a mechanical clamp located between the β -strands A' and G (blue ribbons). The cohesin I module has a 9-stranded β -sandwich jellyroll fold with 2 predicted mechanical clamps located in tandem between the β -strands A-I and A'-I' (blue ribbons). All of the atomic structures were visualized with the visual molecular dynamics (VMD) program (32). (C) The constructs used in our AFM studies. Heteromeric polypeptide fusions of the 3 cohesin I modules were synthesized with a built-in single-molecule marker [i.e., a polypeptide of the titin I27 module (2, 14, 27)]. This module was used here as both an internal control and a single-molecule marker. Constructs of the connecting region of both scaffoldins studied were also made by genetic engineering (bars in A).

mophilic bacterium) and CipC from *C. cellulolyticum* (Fig. 1A) (6). According to our working hypothesis, we expect that the scaffoldin region located between the 2 anchoring points (to the bacterium and to the substrate), i.e., the connecting region, may be subjected to higher mechanical stress (Fig. 1A). Thus, we examined the 3 cohesin I modules for which an atomic structure is available: two of them from the connecting regions [module 7 from CipA (11), c7A, and the first module from CipC (12), c1C], and one from outside this region, i.e., the hanging region [module 2 from CipA (13), c2A].

Mechanical Stability of Cohesins. Mechanical stability was measured by atomic force microscopy (AFM)-based single-molecule force spectroscopy in the length-clamp mode. For single-molecule identification we constructed fusion heteromeric polyproteins in which each cohesin I module was flanked by a marker, a polyprotein of the titin I27 module (see [SI Materials and Methods](#); Fig. 1C; ref. 14). We found that the mechanical stability of the 2 cohesins from the connecting region (c7A: 480 ± 14 pN, $n = 30$; c1C: 425 ± 9 pN, $n = 35$) was far higher than that of the cohesin from the hanging region (c2A: 214 ± 8 pN, $n = 26$), which is comparable to that of I27 (211 ± 3 pN, $n = 237$), all of them probed at the same pulling speed (0.4 nm/ms; Fig. 2). In addition, sequence and mechanical analyses showed that c7A and c1C are representative of the modules present in their connecting regions ([Figs. S1 and S2](#)). Therefore, the mechanical stability (F_u) of the cohesins studied is fully consistent with their putative mechanical function in the scaffoldin physiology. Interestingly, a comparison of the modules from the connecting and hanging regions of the CipA scaffoldin shows that most of the sequence differences are concentrated in the mechanical clamps (44% identity) whereas the cellulase binding region (the dockerin I binding surface, see Fig. 1A and [Fig. S3](#)), is nearly unchanged (97% identity; [Fig. S3](#)).

The results of the present study reveal that cohesins are the most mechanostable proteins reported to date, followed by the I32 module from human cardiac titin, which showed forces of 298 pN at a comparable pulling speed (in proteins, an increase in the pulling speed typically results in higher unfolding forces; ref. 15). Similar high forces have also been reported in two other systems. In the green fluorescent protein (16) the high-force peaks observed when pulling from nonterminal amino acids (548 pN at a pulling speed about one order-of-magnitude faster) can be assumed to be a pure epiphenomenon (i.e., with no physiological relevance) because this protein is not thought to be subjected to mechanical stress. In the adhesive nanofibers (likely proteinaceous) from the extracellular matrix of a fouling diatom, forces of 135–872 pN were reported (at double the pulling speed) (17). Nevertheless, in these experiments the high forces observed are interpreted as being derived from oligomers of parallel molecules in register that are pulled simultaneously. Moreover, it cannot be ruled out that these events may represent the rupture of intermolecular interactions (unbinding) rather than that of intramolecular bonds (unfolding).

Kinetic Parameters of Forced Unfolding in Cohesins. To gain insight into the nature of the extraordinary mechanical stability of cohesin I modules, we analyzed the kinetics of the mechanical unfolding process by using the force-ramp mode of force spectroscopy and Monte Carlo simulations to fit the length-clamp unfolding force histograms (see *Materials and Methods* and Fig. 3). As we found no indication of intermediate states in the unfolding of cohesins, we assumed that the process follows a 2-state kinetic model. The mechanical stability of cohesin I modules correlates well with the height of the energy barrier to the process (related to the unfolding rate at zero force, α_0). The potential widths obtained for cohesins are among the shortest reported to date, indicating that these structures are also par-

Fig. 2. Mechanical stability of cohesin I modules. AFM-based single-molecule force spectroscopy was used in the length-clamp mode (at a pulling speed of 0.4 nm/ms) to measure the mechanical stability of the representative cohesin I modules. The insert in **A** shows the AFM set-up configuration with a stretch of a molecule trapped. (**A**) Three representative force-vs.-distance traces from cohesin I modules with the unfolding force peak events from the I27 marker in black. The last force peak at the end of each curve marks the rupture of the mechanical circuit (i.e., the detachment of the molecule from either the substrate or the cantilever tip) and hence the end of the experiment. For these particular molecules, the unfolding force values were: 562 ± 5 pN for c7A, 430 ± 6 pN for c1C, and 285 ± 6 pN for c2A. After adjusting to the worm-like chain model of polymer elasticity (33, 34), the calculated contour length increments (ΔL_c) for the I27 were 27.3 nm in the upper trace and 28.2 nm in the other two traces. For the c7A cohesin module this value was 49.2 nm and it was 48.2 nm for the c1C and c2A modules. (**B**) Contour length-increment histograms (normalized). We could identify each module individually due to the different increments in contour length of the marker polypeptide. For each construct there are 2 separate populations with characteristic increments in contour length: that of the I27 marker (27.9 ± 0.07 nm, $n = 237$) and that of the cohesin under study (49.3 ± 0.3 nm for c7A, $n = 30$; 48.5 ± 0.2 nm for c1C, $n = 35$; and 48.6 ± 0.2 nm for c2A, $n = 26$). (**C**) Unfolding force histograms (normalized) in which the experimental data are represented by bars. Monte Carlo (MC) simulations of the unfolding force probability are represented by lines. Solid lines represent the parameters that best fit the length-clamp experimental data (see Table 1), whereas dotted lines represent MC simulations using the potential parameters obtained from the force-ramp fittings (see Fig. 3). The dashed line in the I27 force histogram represents MC simulations by using $\Delta x = 0.25$ nm for the I27, as previously reported (35).

ticularly brittle at the experimental temperature. We have also compared mechanical stabilities with the thermal stability of the isolated modules (as determined by the melting temperature, T_m) and found no correlation. As expected, the T_m was found to be related to the optimal growth temperature of the corresponding organisms (Table 1, Fig. S4; see also *SI Materials and Methods*). The absence of such a correlation was also found previously for other modules (2) and is consistent with experiments showing that the thermal and mechanical unfolding pathways seem to coincide at low loading rates but not at the high loading rates used in AFM (which often drive the unfolding process very far from equilibrium) (4).

Steered Molecular Dynamics Simulations of Forced Unfolding of Cohesins. Finally, we performed molecular dynamics (MD) simulations of the mechanical unfolding of cohesins to examine the atomic details of the process and pinpoint the molecular determinants of their mechanical stability (Table S1). It was previously shown that the Ig-like, β -sandwich topology to which the cohesin I modules belong is a platform that can tolerate higher axial mechanical stress than all of the others studied to date (5). The mechanical stability of modules with such topology is often determined by a patch of elements of secondary structure highly localized at the breakpoint and which represent the main mechanical barrier to stretching: the mechanical clamp. This is a secondary structural feature located near the ends of the protein and it is formed by backbone hydrogen bonds between long β -strands (and the associated packing interactions), which are shear-ruptured on pulling from the ends (5).

We used two different all-atom MD simulation methods: the “generalized Born surface area” (GBSA) (18) and the “explicit water molecules” (exW) (19). The GBSA method reproduced the hierarchies of mechanical stability in cohesins correctly, whereas the exW approximation did so more roughly (Fig. 4 and Fig. S5; Table S1). Both methods showed that rather than one mechanical clamp of backbone hydrogen bonds, as previously found in most mechanical proteins, cohesins had 2 tandemly arranged mechanical clamps with a higher number of hydrogen bonds in the modules belonging to the connecting region (A-I and A'-I'; 5 + 5 hydrogen bonds for c7A and c1C cohesins and 4 + 3 for the c2A cohesin) (Figs. S5 and S6 and Table S1). Thus, the mechanical stability of cohesins seems to be directly derived from the number of backbone hydrogen bonds present in their mechanical clamps. The presence of mechanical clamps in protein modules from bacteria suggest that this structural/functional motif is a general principle that is widespread in mechanical proteins. Nevertheless, there is more to protein mechanostability than the mechanical clamp motif. Indeed, in some protein folds the hydrophobic core also contributes to mechanical resistance [e.g., fibronectin type III modules from human tenascin (20)]. There are also exceptions to the shear mechanical clamp topology such as the green fluorescent protein (21) and ankyrin B (22). In addition to this secondary-structure-based elasticity, tertiary [e.g., the solenoid of ankyrin B (22)] and quaternary [e.g., the helical rod of *Escherichia coli* adhesive pili (23) and the myosin II tail (24)] structural elasticities have also been reported.

While this study was in progress, an independent report using

Fig. 4. Atomic details of the process of cohesin stretching obtained through all-atom molecular dynamics simulations. Free MD trajectories and steered MD of cohesins or I27 are shown in [Movie S1](#) and [Movie S2](#) using both the GBSA (18, 37–40) and the exW (19) approximations. (A) Steered MD simulations of cohesin stretching by using the GBSA approximation. Different snapshots at every 200 ps were taken as the starting point of several unfolding trajectories. The averages of the force-distance traces are shown. This approximation reproduces the experimental mechanical hierarchy and the unfolding forces are in the same order of magnitude as experimental data ($F_{I27} = 370 \pm 80$ pN; $F_{C7A} = 710 \pm 120$ pN; $F_{C1C} = 630 \pm 70$ pN; and $F_{C2A} = 470 \pm 80$ pN). (B) Steered MD simulations of cohesin stretching using the exW approximation. As previously reported (19), this approximation yields unfolding forces one order-of-magnitude larger than the experimental results. Also, the relative order of the mechanical stabilities for the cohesin modules under study is less well reproduced than with the GBSA approximation at the same pulling speed ($F_{I27} = 910 \pm 50$ pN; $F_{C7A} = 1360 \pm 50$ pN; $F_{C1C} = 1610 \pm 30$ pN; and $F_{C2A} = 860 \pm 50$ pN; [Table S1](#)). In addition, 2 lower speeds were used, separated by an order of magnitude. The 3 different pulling speeds are represented from left to right (1, 0.1, and 0.01 Å/ps, respectively). (C) Histograms of the unfolding force peaks from several trajectories obtained through the GBSA approximation at 1 Å/ps are shown. (D) One trajectory obtained by the exW approximation at 0.01 Å/ps is presented.

1. In terms of protein nanomechanics, we have identified the most robust mechanical protein structures found to date: the cohesin I modules from the connecting region of scaffoldins. These modules represent the current upper limit of this important biophysical property, and its mechanical topology seems to be an extension of a common principle that involves the presence of shear mechanical clamps of secondary structure fastening the ends of mechanical proteins. It is tempting to speculate that the existence of this feature, previously described in adhesion proteins from metazoans and now reported in those from bacteria, may represent an example of convergent evolution toward a common structural solution to a similar functional problem (i.e., mechanical resistance). We suggest that this structural/functional motif may be a general principle in biology to achieve the necessary mechanostability that mechanical proteins require to maintain their structural integrity *in vivo*. We also present experimental demonstration of the validity of coarse-grained simulations to predict mechanical stability [this is particularly important because a massive PDB-wide survey using this method has recently been reported (25)]. However, additional experiments will be necessary to further test the robustness of these models.
2. In terms of cell biology, the remarkable mechanical stability of scaffoldins in the connecting region raises the possibility that this trait may have been adaptive (i.e., subjected to selection pressure), representing an adaptation to resist extremely high axial forces (which would prevent the detachment of cellulases), and it suggests that these proteins may

have an additional role as mechanical scaffolds. Moreover, we propose that mechanical stability could serve as a useful parameter to test the vast number of architectural models of supramolecular (quaternary) structure proposed for the overwhelming variety of scaffoldins (i.e., to distinguish between connecting and hanging modules).

3. In terms of protein engineering and materials science, the extremely high mechanostability of cohesin I modules provides the basic building blocks for the introduction of this variable whenever it may be necessary in protein design for biotechnological or nanotechnological applications. Grafting of mechanical clamps has already been reported (26). In biotechnology, the control of this variable may permit more efficient cellulosomes to be designed for the industrial biodegradation of cellulose (the most abundant organic compound on Earth and hence a promising renewable source of fuel products such as bio-alcohol). Indeed, in the light of our data, it is now possible to select not only thermostable scaffoldins (to work at higher temperatures) but also highly mechanostable modules (e.g., likely resistant to strong stirring). In bio-inspired nanotechnology, the “hyperstability” of some cohesin modules (both thermal and mechanical) is an attractive property that could be used to design sturdy bio-scaffoldins (6). Such properties may be exploited for the spatiotemporal coordination of specific enzymatic activities to achieve more efficient degradation of recalcitrant or complex substrates.

The results reported here raise many questions that should be addressed in future studies. For instance, it is important to know

to what extent high mechanical stability is a common property of adhesion proteins from motile unicellular organisms, and whether the mechanical clamp motif is present in other bacterial proteins (i.e., both adhesion proteins and other proteins putatively subjected to mechanical stress). In addition, our mechanical hypothesis could be tested by analyzing the nanomechanics of additional cohesins from both the connecting and hanging regions of other scaffoldins, as well as the possible effect of cellulases on cohesin mechanostability. Furthermore, the putative adaptive character of this trait should be tested, measuring both the forces that are held by the connecting and hanging regions in vivo, and the mechanostability of the linkages in the scaffoldin system.

Materials and Methods

Polypeptide engineering was performed with a vector described previously (27) and the scaffoldin constructs were PCR cloned de novo from bacterial genomic DNA. Due to the high degree of conservation among its cohesin I modules (28), the *C. thermocellum* CipA scaffoldin construct was sequenced by using a method based on nested deletions. The AFM used in this study is a

slightly modified version of a previously described home-made instrument (29) with added imaging capabilities (30). Previously established methods were followed to measure thermal stability (31) and the 3 types of MD simulations used here: coarse-grained (25) and all-atom with implicit (18) and explicit (19) solvent.

A full description methods and any associated references are available in the *SI Materials and Methods*.

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