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Extreme mechanical stability in protein complexes

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Recently, non-covalent protein complexes and folds with extreme mechanical stabilities have been discovered. Various extracellular adhesin proteins of gram-positive bacteria exhibit complex rupture forces ranging from 800 pN in the case of cellulolytic bacteria to over 2000 pN withstood by pathogens adhering to their hosts. Here, we review and assess the mechanics of such systems, and discuss progress, as well as open questions regarding their biological function, and underlying molecular mechanisms — in particular the role of increased interaction lifetimes under mechanical load. These unexpected extreme strengths open an unchartered range of protein mechanics that can now be routinely probed by atomic force microscopy-based single-molecule force spectroscopy.

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Introduction

Protein–protein interactions show a great variety of mechanical properties. Recent discoveries have revealed a greatly increased range of maximum mechanostability of protein folds and complexes. Interactions primarily found in the extracellular space, often responsible for anchoring bacteria to a desired target, show previously unanticipated mechanical strength. Some systems even approach to the force required to break a covalent bond. Recent reviews discuss the cases of pathogen adhesion proteins with extreme mechanical strengths [1–3].

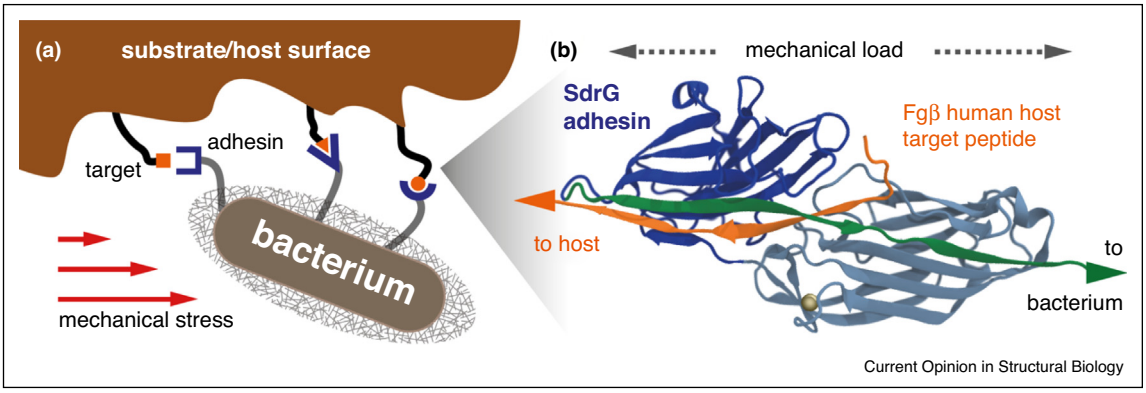
Forces in excess of hundreds of pN are particularly suited to be studied by Atomic Force Microscopy (AFM)-based single molecule force spectroscopy (SMFS) in which an AFM cantilever of known spring constant is used to measure forces required to dissociate biomolecular interactions or to unfold proteins [4]. Forces exceeding 1 nN can almost exclusively be studied in AFM-SMFS.

In recent years a number of systems from extracellular domains, have shown extreme, unexpected mechanostabilities to be discussed herein. Primarily adhesion protein complexes that anchor a bacterium to a desired target such as its host in case of pathogens, or to a substrate that cellulolytic bacteria digest. To set the scale: Biotin:Streptavidin is a standard pull down and tethering system of extremely high affinity (K_D∼fM) . It is generally considered mechanically strong as used in optical, magnetic, or acoustic tweezers based assays and only dissociates at around 150 pN at force loading rates of 1e4 pN/s [5]. Here, we consider systems highly mechanically stable that approach or exceed 500 pN in rupture force at similar force loading rates. Among the first of which from recent years were the cohesin-dockerin type III complexes that anchor cellulolytic bacteria to their cellulose substrate [6,7]. These have proven to be reliable handles for high stability force spectroscopy [8–11].

Pathogen adhesins

Extreme mechanics are found predominantly in the extracellular space, notably when responsible for anchoring bacteria to their respective targets, as shown in Figure 1a. Gram-positive adhesins, such as the prototypical SdrG, a so called MSCRAMM (microbial surface components recognizing adhesive matrix molecules) from *Staphylococcus epidermidis* bind to human adhesive matrix proteins, especially fibrinogen. These systems target a short peptide on the order of 10–15 amino acids, such as the N-terminus of the fibrinogen β chain (Fgβ) in the case of SdrG, Figure 1b. The force required to sever this complex between the mere 40 kDa SdrG and the short peptide at force loading rates around 1E5 pN/s is larger than 2000 pN, a clear record in complex mechanostability, that is only superseded by some of its direct homologs from similar bacteria [12••,13•,14••,15]. These results hold both *in vitro* and *in vivo* on live bacteria. Even in molecular dynamics simulations *in silico* these extreme strengths have been reproduced, albeit shifted at higher force loading rate in agreement with single-barrie unbinding models such as Bell–Evans [16] and Dudko–Hummer–Szabo [17]. Currently, the AFM is the only instrument capable of routinely exploring this range [4], as seen in Figure 2a. It remains unclear what exact physiological forces actually act on such adhesins, as well as if, and if yes how, they coordinate collectively to achieve strong adhesion to their host.

Figure 1



Mechanically challenging environments require specific and strong tethering of bacteria to their substrates and, in the case of pathogens, hosts. **(a)** Gram-positive bacteria covalently couple receptors (blue) to their peptidoglycan layer via a sortase motif. These can recognize substrates for the bacteria, for example, cellulose as in the case of some *Ruminoclostridia*, or host molecules as in the case of *S. epidermidis* or *S. aureus*. The attachment must have evolved to withstand considerable forces propagated from the hydrodynamic stress that act on the bacterium, for example, in shear flow, to keep it bound to its target.
(b) The crystal structure of one of the recently described strongest receptor–ligand systems pathogen surface presented SdrG (PDB 1R17 [48], blue) and its human peptide binding partner Fgβ (orange) show how the specific arrangement of two binding partners enables a native shear geometry of receptor and ligand (large arrows).

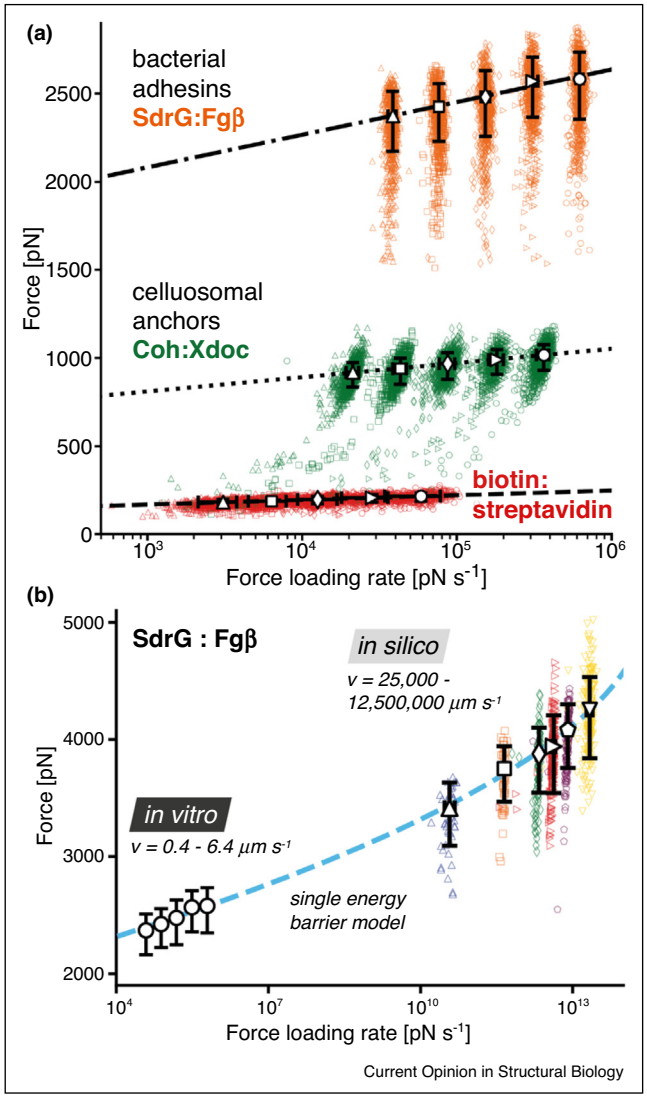
Similar bacterial adhesins have shown rupture forces much larger than 1000 pN [18]. More recently even in systems such as the alpha-helical Protein A of *Staphylococcus aureus* binding von Willebrand factor 2 nN strong forces have been reported [19]. Some bacterial adhesins, such as the so called thioester domains [20] even target their hosts with covalent bonds, attaching with an isopeptide bond in a mechanically steered manner [21]. Notably, for the adhesin ClfA from *S. aureus* two binding sites have been proposed that are regulated by force application to the complex [22•]. It could also be argued that binding modes of such a system may switch to enhance attachment stability.

Crucially such investigations can only be made with a fully covalent surface anchoring strategy to ensure that one can observe the high force rupture of the ultrastable adhesin system. Otherwise, the weakest non-covalent link in the attachment chemistry would break before the complex can rupture [23]. One could even speculate that in rare cases the covalent surface attachment chemistry breaks, as it may only be marginally stronger than the pathogen adhesion systems under investigation. Studies on extreme stability protein complexes are only possible because covalent coupling strategies can now be employed routinely. However, it is crucial that the coupling is site-specific through appropriate tags to ensure a single, unambiguous force application geometry, as discussed in the next section. Covalent, yet ambiguous coupling such as targeting many of the primary amines in a protein cannot provide site specificity. It may even create a multitude of force loading geometries each inducing different responses that cannot be disentangled [24].

In the case of understanding ultrastable pathogen adhesion, a molecular basis for this strength is required [25]. On a single molecule-basis molecular dynamics simulations (MD) [26] are powerful tools to unravel underlying molecular mechanisms through the 'computational microscope'. Recent work has shown exceptional correspondence between experiment and simulation [27,28•]. It is essential that simulation models are complete and include adjacent protein sequences in the case of peptides or other features, as, for example, introduced by surface coupling linkers [29]. Figure 2b shows one example of this agreement within the same theoretical framework, which instills confidence in the accuracy of MD simulation models — that are in turn critically dependent on the accuracy of force fields used. At least for these very rigid, high-force systems, MD simulations have provided an experimentally confirmed, accurate description.

A mechanism governing this extreme stability has been proposed for the SdrG adhesin and its homologs, using the interplay between molecular dynamics simulations and experimental mutants. At the core of this unusual mechanostability a confined backbone hydrogen bond shear geometry between human target peptide and pathogen adhesin is formed. As this network mainly relies on the target peptide backbone, it ultimately renders this extraordinary strength virtually independent of target peptide side chains, and thus sequence. This hypothesis is supported by a pathogen adhesin ClfB from *S. aureus* that can achieve more than 2000 pN in rupture force when binding a target sequence composed entirely of glycines and serines — meaning essentially no large or

Figure 2



Recently characterized receptor ligand systems have greatly increased the range of accessible maximum rupture forces.
(a) The dynamic force spectra shown exemplify this. Biotin: Streptavidin in its standard, N-terminally tethered geometry ruptures around a mere 150 pN, data shown here from monomeric Streptavidin [49,50]. The CohesinE:Xmodule-dockerin systems from cellulosomal bacteria already extended the range of forces accessible with a protein–protein receptor ligand systems to more than 700 pN [28•]. Finally, bacterial adhesins like SdrG binding the Fgβ peptide, and their homologs allow probing of a vastly greater range of forces up to 2500 pN, data from [12••]. Forces larger than 1000 pN can now be probed routinely allowing investigations on new, extremely stable systems.
(b) Molecular dynamics simulations *in silico* due to limited simulation time operate at much higher pulling velocities and thus force loading rates than experimental *in vitro* force spectroscopy. Yet, within the Dudko-Hummer-Szabo model [17] they can be fit to a single set of parameters – an excellent correspondence between experiment and simulation, here shown for SdrG: Fgβ.

special side chains. Partially, this mechanism may explain how pathogens can adhere to such a large variety of targets. When a target peptide is confined in the backbone-backbone shear geometry it automatically renders these interactions extremely mechanically stable [12••].

Small Ig-like folds called B domains propagate mechanical stress from the adhesins discussed above to the bacterium. Recently, these were established as the strongest protein fold by a large margin [30,31], unfolding at over 2000 pN. Additionally, it was elucidated how calcium regulates their stability and proposed function as molecular shock absorbers [13•]. Intriguingly, the mechanically strongest protein complexes, such as SdrG are barely strong enough to unfold their neighboring B domains: the mechanically most resilient protein fold by a large margin.

Geometry determines strength

One would intuitively suspect that thermodynamics and mechanics of such extremely strong interactions would be related, yet this is not necessarily true. Instead, force application geometry and propagation through a protein complex have been identified as crucial factors [29,32]. This is best exemplified by the low affinity of bacterial adhesins (K_D in the micromolar range), when contrasted with their extreme mechanics.

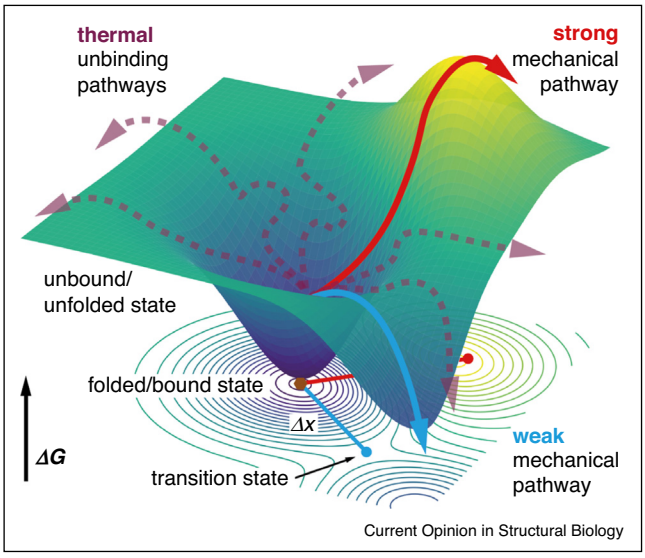
The force propagation pathways critically determine complexe strength [32]. To assess a system's mechanical properties, it is vital to probe it in its native, physiological direction of force application from the correct N-terminus or C-terminus. This effect is drastically illustrated by the SdrG:Fgβ system as seen in Figure 4a. The native pulling configuration reaches over 2000 pN in rupture force. However, when the peptide is pulled non-natively, that is, from the N-terminus instead of the natively loaded C-terminus, rupture forces plummet to a mere 60 pN. Conversely, when loading the adhesin non-natively from its N-terminus, and the target peptide natively from its C-terminus rupture forces reduce to about 250 pN [33].

Another striking recent example extends this to small molecules. Biotin dissociating from its streptavidin binding pocket is susceptible to force propagation changes [29,34]. Similarly, the tetramer subunits allow for distinct tethering geometries of streptavidin depending on its anchor point relative to the position of the bound biotin [35].

These results tie into the catch-bonding idea [36,37], in which most generally speaking interactions increase their bound lifetime under force, that is, profit from the force up to a certain level as it makes them bind more tightly. While high complex rupture forces are in themselves not

indicators of catch bonding, such a high force combined with fast off-rates (short complex lifetimes in bulk) strongly hint at catch bonds – although the final demonstration is still missing for the bacterial adhesins here. There is some evidence in this direction for the adhesin ClfB from *S. aureus* [38•].

Figure 3

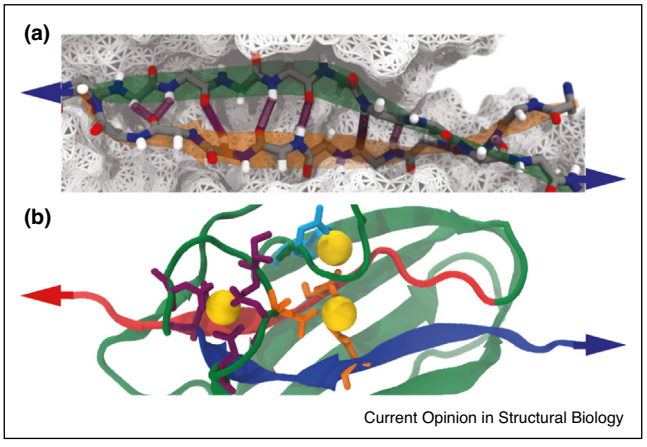


Mechanical opposed to thermal unbinding in a schematic energy landscape.
When a complex unbinds, the rough potential energy landscape is probed by thermal excitations. Depicted here are only several of a multitude of unbinding paths. Since the probability for a particular path is exponentially weighed by the height of the transition state barrier (see marked as distance to the transition states Δx in red and blue at the bottom for the mechanical pathways), paths with high barriers are very unlikely to contribute to thermal unbinding in the ensemble. The system samples the 'valleys'. An external force acting on the complex selects a direction in the energy landscape (this is done, for example, by the choice of the attachment points on the molecular components). Thermal fluctuations are biased by this force. If the direction of this force acts in the direction of the most probable thermal unbinding path, the likelihood for overcoming the unbinding barrier is increased and the bond lifetime is reduced. One would call this a slip bond, for example, in the case of a weakened unbinding pathway in cyan here. If another direction is chosen for the force to act on the complex, a higher barrier is encountered by the complex on its unbinding path (red). As a result, the most probable unbinding force will be much higher than in the case of the slip bond. On this pathways, if the force is kept constant, for example, at the level that dissociates the the slip bond pathway, the thermal fluctuations are biased away from the thermal transition barrier and as a result the lifetime of the bond exceeds the thermal lifetime. One would call this a catch bond. Since many escape paths exhibit higher barriers than the thermal paths when loaded in the right geometry, we expect the number of potential catch bonds to be very large, possibly even exceeding the number of slip bonds. In other words, from a protein mechanics perspective many – especially low affinity interactions – may be catch bonds. The higher a most probable unbinding force of the complexes is, the smaller the contribution of thermal fluctuations to the unbinding process, and thus the more pronounced a catch bond behavior of that system.

SMFS only probes a single unbinding geometry defined by the tethering points from which force is applied, enforcing a path along the energy landscape that may require overcoming a high energy barrier, or conversely just a low one. Thermal unbinding, as measured in bulk affinity assays usually has many more open pathways and geometries available to unbind. This discrepancy may render some mechanical geometries very resistant to force, as the receptor–ligand complex is geometrically constrained to dissociate against a very high activation energy barrier in its energy landscape.

Provocatively, one could propose that 'every bond can be a catch bond' if one can find a pulling geometry that renders the complex mechanically strong along a certain reaction coordinate [39]. Such pathways are unfavorable in the complex energy landscape [40] as illustrated by the examples above and in Figure 3. Because of their high energy barrier, they will not be favored in thermal unbinding. However, crossing the high energy barrier

Figure 4



Molecular basis of extreme mechanical strength in proteins and protein complexes.
(a) Hydrogen bonds are at the core of extreme bacterial adhesin strength. Confined in a shear geometry the backbone–backbone hydrogen bonds (purple) between the antiparallelly aligned peptide (orange) confined by the adhesin (white surface, green β-strand) cooperatively rupture in a single step, making them collectively withstand forces of over 2000 pN. Force application in the native geometry from C-termini (blue arrows).
The adhesin's structure prevents sequential breaking of hydrogen bonds, which happens when the force application geometry is changed to a non-native pulling geometry, by tethering the peptide from its N-terminus. In this case the rupture forces are merely 60 pN, as the hydrogen bonds now sequentially break in a zipper mode.
(b) Similar, extreme stability with unfolding forces of over 2000 pN is achieved in the MSCRAMM B1 domain fold shown in green. The mechanism here rests on the coordination of three Calcium ions (yellow, coordinating amino acids shown as sticks) that are aligned across the parallel N-(red) and C-terminal (blue) β sheets. Calcium removal by chelation results in much weaker rupture forces of only about 600 pN. An exact molecular mechanism of how this coordination drives the stability has yet to be found.

pathways can be enforced by force application geometry. Conversely, these high-barrier pathways have longer bound lifetimes under force than permitted by the thermal, bulk off off-rate — resulting in a catch bond. In the case of pathogen adhesins, this behavior likely evolved to withstand high mechanical stress, while allowing for flexible unbinding with a thermal bond lifetime on the order of minutes in stress-free environments. Potentially, catch bond behavior could even be rationally designed by engineering a high mechanical unbinding energy barrier yet permitting fast thermal 'off-rates of a protein complex.

Furthermore, in this line of argumentation, there must be a crossover between mechanical and thermal (kinetic, zero-force) unbinding, especially when unbinding forces are high and affinities low. The shape of that transition from thermal to mechanical unbinding remains to be characterized, but should be accessible with long observation times in very slow force ramp and constant force assays [41,42].

Conclusion and outlook

While non-specific polyprotein pulling, in which proteins of interest and fingerprint domains are expressed as repeat constructs and allowed to non-specifically adsorb to an unfunctionalized cantilever, can achieve high forces successfully [30,43], the nN force regime is difficult to access with these experiments.

Systems of extreme mechanical stability are ideal handles and targets of further study for AFM-SMFS, which due to its rather large baseline noise is capable [44], yet inherently limited in studying comparatively low-force systems. A new range for force spectroscopy, exceeding 1 nN is now routinely accessible when using the new handles described here, sufficient to unfold almost any non-covalently linked protein [31] or receptor-ligand complex [45]. Especially the pathogen adhesins described here are useful as they only require a short (<15 amino acid) peptide target on a protein of interest and are straightforward to produce. These new force regimes preserve AFM-SMFS' role as a key technique for protein mechanics. Nevertheless, these mechanics must be better contextualized with *in vivo* measurements to gauge what form and magnitude of mechanical stresses systems investigated are subject to physiologically.

Practically, the extreme mechanics discussed here open new possibilities to be rationally engineered, for example, to be used in tough protein hydrogels [11,46,47]. Extracellular domains in challenging mechanical conditions evidently evolved to securely anchor bacteria with extreme resilience to mechanical stress. Thereby, they open up a new biomolecule mechanostability regime, an unexplored force range, an undiscovered country of protein mechanics to be charted in the near future.

Conflict of interest statement

Nothing declared

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- •• of outstanding interest

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