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Previews

Force-induced unfolding of an antibiotic-bound outer-membrane protein

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In this issue of *Structure*, Ritzmann et al. characterize the unfolding of the β-barrel assembly machinery component BamA with single-molecule force spectroscopy and reveal how an antibiotic changes BamA's mechanical properties and inhibits its activity. This work helps us understand the effects antibiotics have on Gram-negative outer membrane proteins.

Darobactin is a newly discovered antibiotic that selectively kills pathogenic Gram-negative bacteria (Imai et al., 2019). It does so by mimicking the recognition signal of native substrates of the β-barrel assembly machinery (BAM) (Kaur et al., 2021) that is responsible for the folding and insertion of β-barrel proteins into the outer membrane. Recent structural studies revealed that darobactin binds to the lateral gate of the BAM subunit BamA with high affinity, outcompeting cognate substrates and therefore inhibiting its essential insertase activity (Kaur et al., 2021). A question arises from this work: how does binding of darobactin affect the mechanical and thermodynamic properties of BamA considering its role in protein folding? Atomic force microscopy (AFM) single-molecule force spectroscopy (SMFS) is a powerful technique to address this question.

AFM consists of a solid probe with a very sharp tip (~10-nanometer diameter) that touches a sample (Viljoen et al., 2021). The probe is connected to a soft cantilever, from which a laser beam is reflected onto a photodiode. Forces between the tip and the sample cause a bending of the cantilever, which changes the laser's path that is registered by the photodiode. If certain physical characteristics of the cantilever are known, the magnitude of forces between the AFM tip and the sample can be precisely quantitated with piconewton sensitivity. SMFS works by picking up single molecules with the AFM tip. If the other end of a molecule is securely anchored to a sample surface, further displacement of the tip away from the surface will result in a stretching of the molecule until an unbinding event occurs, e.g., bonds between the molecule and the tip are broken, or the molecule ruptures. The recorded data are so-called force-distance (FD) curves. Careful analysis of these data allows the filtering of curves showing the stretching patterns of single molecules only, therefore giving us the term SMFS. When stretching proteins, one can often observe sequential force peaks representing the unfolding of single domains, which allows determining their relative contributions to the overall protein stability under tensile stress.

In 2000, Filipp Oesterhelt, Daniel Müller, and colleagues provided proof of concept that AFM can be used to pull single bacteriorhodopsin proteins in purple membrane patches until they were fully unfolded (Oesterhelt et al., 2000). In their experiments, purple membranes were first adsorbed to flat surfaces. Owing to the extremely high resolution of a few nanometers afforded by AFM topographic imaging, they could localize bacteriorhodopsin trimers within the purple membrane. They then brought an AFM tip in contact with a single bacteriorhodopsin molecule for the relatively long duration of 1 s while exerting an arbitrary pressure so that strong non-specific adsorption occurred between the terminal regions of the protein and the tip. Subsequent retraction of the tip gave rise to well-resolved FD curves detailing adhesive events in approximately 15% of the time. After pulling a single protein out of the purple membrane, subsequent topographic imaging also clearly showed its absence from its original position in the membrane. Analysis of the FD curves revealed the unfolding events, which demonstrated that transmembrane helices are anchored by 100–200 piconewtons of force and that the different helices displayed individual unfolding pathways. This work opened the door to a slew of breakthroughs in characterizing the mechanical properties of integral membrane proteins.

The group of Daniel Müller has also been leading the field on the nanomechanics of integral membrane proteins located within the outer membrane. In 2009, they used SMFS to fully unfold the β-barrel outer-membrane protein OmpG from *Escherichia coli* for the first time (Sapra et al., 2009). In follow-up work two years later, they used SMFS to study the refolding of OmpG and its reinsertion into a lipid membrane (Damaghi et al., 2011). Now, in this issue of *Structure*, Ritzmann et al. (2021) have unravelled how darobactin affects the mechanical, kinetic, and energetic characteristics of BamA (Figure 1). By pulling on BamA's N terminus, they succeeded in sequentially unfolding the five polypeptide transport (POTRA) domains followed by the β-hairpins of the transmembrane β-barrel. When comparing the FD curves obtained for BamA with those from the protein treated with darobactin, they observed that the tension required to unfold the β-hairpins is higher in the presence of darobactin while that for the POTRA domains remains unaffected, indicating that the antibiotic increases the mechanical stability of the insertase. Next, they asked how darobactin affects the free-energy landscape of BamA unfolding. To answer this question, they used SMFS in dynamic force spectroscopy (DFS) mode, in which the protein was unfolded under varying force loading rates, which was

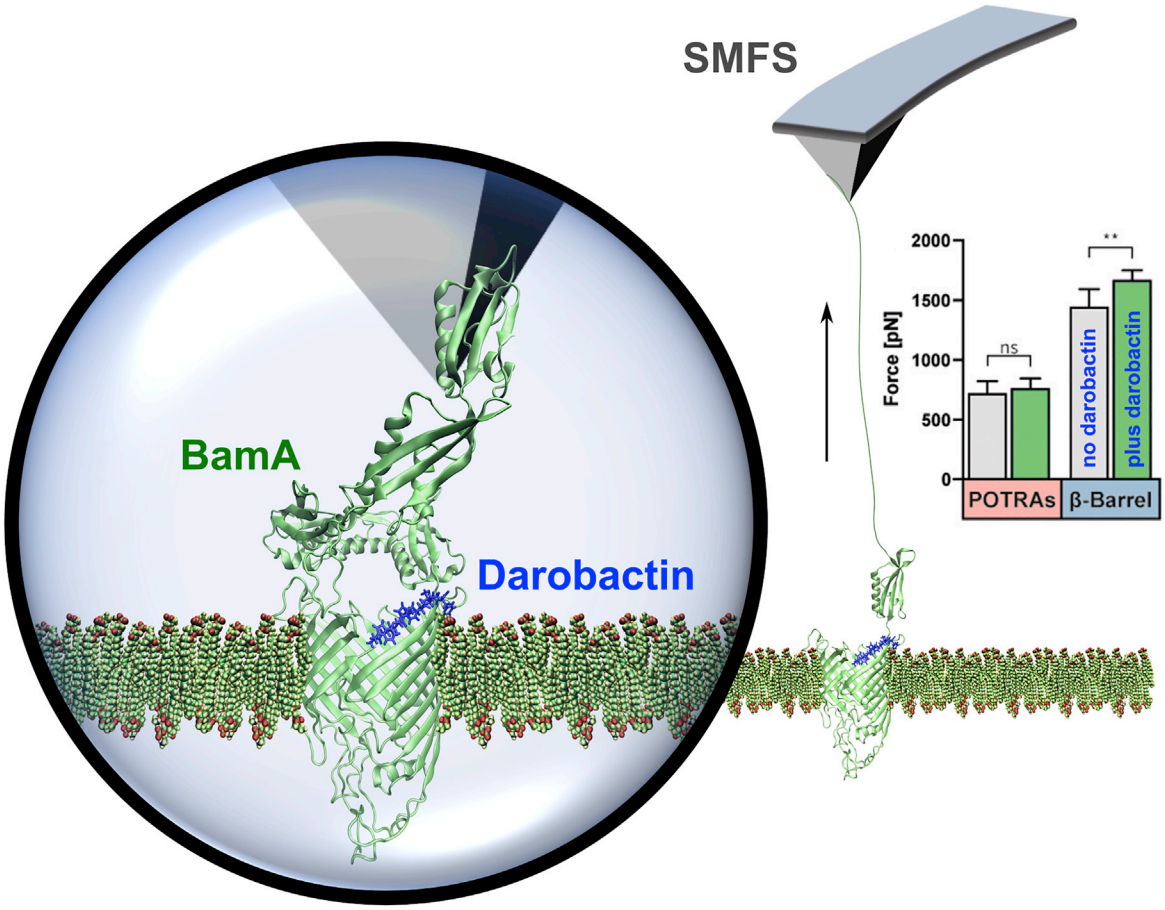

Figure 1. SMFS helps unravel how an antibiotic causes changes in the structural stability of its outer membrane protein target
The bar graph showing the effect on the tensile force required to unfold POTRA or β-barrel domains in BamA in the presence or absence of darobactin was adapted from Ritzmann et al. (2021).

accomplished by changing the pulling speed. By fitting the resulting DFS data (unfolding force versus loading rate) with a suitable theoretical model, they were able to quantify the thermodynamic parameters of BamA unfolding in the absence or presence of darobactin. They found that the antibiotic widens free-energy wells of the β-barrel domain, lowers the unfolding rates of this segment, and increases its energetic stability. On the other hand, kinetic and energetic properties of the POTRA domains were less affected by darobactin. Finally, they investigated the effect of darobactin on the elasticities of the different domains in BamA and found that in the presence of the antibiotic, the stiffnesses of both POTRA and β-barrel domains are reduced. In summary, these findings support the view that darobactin considerably increases BamA's mechanical stability, which is most prominent for the β-barrel domain. These changes unbalance the finely tuned properties of the BamA subdomains necessary for the folding and funneling of substrate β-barrel proteins into the outer membrane. This exciting work illustrates the usefulness of SMFS as a tool for analyzing the effects that antibiotics have on their integral membrane protein targets, which are notoriously difficult to study. This new knowledge about BamA, together with mutagenesis approaches and molecular dynamics simulations, could help facilitate the rational design of novel BAM inhibitors with increased therapeutic potential that are direly needed in the context of increasing antibiotic resistance among bacterial pathogens.

A grand challenge for the future is to perform these kinds of experiments on living microbial cells. During the past decade there has been major progress in this direction. For example, Alsteens and colleagues found that pulling and unfolding single Als adhesins on the surfaces of living yeast cells promoted their clustering into nanodomains on the cell surface (Alsteens et al., 2010). Unfolding of the adhesins resulted in rapid hydrophobic and slower amyloid interactions in extended tandem repeats with neighboring adhesins. Hence, the clustering effect could be attributed directly to unfolding rather than to a remodeling of the yeast's cell wall. This study highlights how pathogenic yeasts like *Candida albicans* use mechanical stimuli to induce clustering of their surface adhesins and ultimately strengthen the adhesion to target substrates. Another class of microbial adhesins for which the impact of unfolding on function has been unraveled by AFM are the *Staphylococcus aureus* surface-adhesion proteins. One example is SasG (Formosa-Dague et al., 2016), which is responsible for intercellular

adhesion during the early accumulation phase of biofilm formation. Single-cell force spectroscopy (SCFS)—where a single bacterial cell is first caught by a special adhesive AFM probe, and the resulting bacterial probe is used to force probe another single cell—showed that SasG mediates interbacterial adhesion through zinc-dependent homophilic bonds involving G5-E repeat domains. Unfolding of these domains gave rise to a sawtooth pattern in the FD curves, from which the forces of unfolding could be determined. Notably, the individual domains were resilient to tensile stress, unfolding at relatively high forces of ~500 pN. These results highlight how a bacterial adhesin might be adapted to maintain intercellular contacts amidst physiological shear forces. With regard to the interactions of antimicrobials with their targets, AFM SMFS and SCFS have shown their value for studying the inhibition of surface adhesins. To supplement current antibiotics, anti-adhesion therapies are attractive because they typically do not target processes that are directly essential to a pathogen's survival but rather impede their ability to efficiently colonize a host, thereby facilitating natural clearance of the infection by the immune system while minimizing resistance acquisition. By way of example, AFM SMFS and SCFS studies recently demonstrated the potency of a peptide originating from β-neurexin as a competitive inhibitor of the homophilic adhesive activity of the *S. aureus* serine aspartate repeat adhesin, SdrC (Feuillie et al., 2017). Homophilic interactions between SdrC adhesins also contribute to intercellular adhesion, and, therefore, the peptide holds promise as an inhibitor of *S. aureus* biofilm formation, with potential for clinical applications. We foresee an increasing role for AFM force spectroscopy methodologies in the delineation of antimicrobial mechanisms of action, especially for those with targets found on the surfaces of microbes and also for the analysis of the mechanical effects of inhibition in general due to the sensitivity of these techniques.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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