

Mass Spectrometry informs the structure and dynamics of membrane proteins involved in lipid and drug transport

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Abstract

Membrane proteins are important macromolecules that play crucial roles in many cellular and physiological processes. Over the past two decades, the use of mass spectrometry (MS) as a biophysical tool to characterise membrane proteins has grown steadily. By capturing these dynamic complexes in the gas phase many unknown small molecule interactions have been revealed. One particular application of this research has been the focus on antibiotic resistance with considerable efforts being made to understand underlying mechanisms. Here we review recent advances in the application of MS that have yielded both structural and dynamic information on the interactions of antibiotics with proteins involved in drug efflux and bacterial cell envelope biogenesis.

Highlights

Mass spectrometry-based methods can characterise both the structural and dynamic impact of small molecule interactions with membrane proteins

Assembly of efflux systems can be monitored in terms of antibiotics binding

The methods described here can be used for screening membrane protein-protein interaction inhibitors

Keywords: Native Mass Spectrometry, Hydrogen-Deuterium Exchange Mass Spectrometry, Antibiotics resistance, bacterial cell envelope, drug efflux

Introduction

Membrane proteins carry out a plethora of vital functions in many diverse biological processes, including energy generation, signalling between cells, transport of ions and molecules and enzymatic catalysis. In humans, these proteins are regularly implicated in disease, as such they represent targets of approximately 60% of all available drugs in the current market [1-4]. Additionally, membrane proteins are often involved in host-pathogen interactions. Understanding interactions at cell membranes will therefore aid the development of the next generation of antimicrobial agents. These agents will be important for combating infectious diseases, not least the current COVID-19 pandemic [5], but also the increasing number of drug-resistant bacterial infections [6].

To improve drug design strategies a clear understanding of the factors that determine the structure and dynamics of membrane proteins is crucial [7]. Recent improvements made in major biophysical techniques, X-ray crystallography, cryo-electron microscopy (cryo-EM) and nuclear magnetic resonance spectroscopy, over the past decade are allowing membrane protein structure determination at unprecedented rates (<http://blanco.biomol.uci.edu/mpstruc/>) [8-12]. Despite this rapid progress, it remains challenging to assess the impacts of lipids and drugs on these dynamic structures. To address this concern, mass spectrometry (MS) has emerged as a complementary biophysical technique that can provide information on the structure, dynamics, and interactions of membrane proteins in the context of small molecules. Several recent excellent reviews describe the progress in MS for membrane protein research [13-22], our focus is on applications directed at understanding antibiotic resistance mechanisms.

While much attention is on the current pandemic, more than 70% of COVID-19 patients have been receiving antimicrobial treatments [23]. This will likely intensify the spread of antibiotic resistance, leading in turn to increased morbidity, mortality rates and health care costs. The most prominent antibiotic-resistant infections to date are caused by the so-called 'ESKAPE' pathogens, classified as priority 1 pathogens by the World Health Organisation. The majority of the ESKAPE pathogens are Gram-negative bacteria, including the carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and cephalosporin and carbapenem-resistant *Enterobacteriaceae* [24]. These Gram-negative bacteria are intrinsically resistant to many antibiotics attributed to both the low permeability of their outer membranes (OM) and the high expression of efflux pumps that export antibiotics out of the cell [25]. A detailed understanding of these resistance mechanisms, and their interactions with antibiotics, is not only critical for the design and development of new therapeutic strategies but also imperative for combatting multiple life-threatening pathogens.

Enormous progress has been made over the past two decades in understanding the transport and assembly of OM components and efflux pumps. Comprehensive reviews cover advances in understanding the biogenesis of the outer membrane [26-30] and of efflux pumps [31,32]. Here, we review recent progress in MS-based methods directed specifically at OM biogenesis and mechanisms of efflux pumps highlighting attributes of the approach for probing interactions between membrane proteins with substrates and antibiotics. We also discuss future prospects for MS in ejecting complexes from native-like membrane environments and in identifying small molecule inhibitors within membrane proteins assemblies.

Lipid transport – the LPS transport system

Considering the first line of defence for Gram-negative bacteria, the asymmetric OM layer, which consists of phospholipids and lipopolysaccharides (LPS) in inner and outer leaflets, respectively. The

presence of this LPS endotoxin is largely responsible for the impermeability of the membrane to hydrophobic molecules such as antibiotics, dyes, and detergents. LPS is a glycolipid comprising three essential portions: lipid A, core oligosaccharide and O-antigen polysaccharide. The lipid A-core oligosaccharide complex ('rough' LPS) and the O-antigen units are synthesised independently in the cytoplasm and then transported to the periplasmic side of the inner membrane (IM) by MsbA [33] and Wzx [34], respectively. Mature (or 'smooth') LPS is transported from the IM to the outer leaflet of the OM through a membrane-to-membrane protein bridge [35], comprising three sub-complexes: the ABC transporter LptB2FG with LptC in the IM, the periplasmic LptA oligomer, and the OM LptDE heterodimer [26,36] (Figure 1a). With the exception of LptB, the other 6 Lpt proteins contain a so-called β -taco domain (Lpt fold) [37,38]. LptC is known to position between the transmembrane domains of LptF and LptG, and the β -taco domain interacts with LptA [39]. LptA is an oligomeric protein [40,41] forming the periplasmic bridge with the C-terminal domain of LptC and the N-terminal region of LptD [42]. Interestingly, the stoichiometry of LptA oligomer *in vivo* remains an open question. LptD has a β -barrel with a lateral opening, which thought to be the exit gate for LPS [43]. LptE is inserted in the lumen of the β -barrel and act as a plug that interacts with the hydrophilic portion of LPS [44]. This Lpt machinery has been likened to a PEZ candy dispenser which drives LPS upwards through (LptB2FGC) constantly pushing (LPS) towards the top. When LPS is ejected from the top it is passively inserted into the OM through LptDE [45].

Given the importance of the Lpt system, much research has been devoted to the discovery of protein-protein interaction (PPI) disruptors. These include the peptidomimetic murepavadin, which was shown to specifically interact with *Pseudomonas aeruginosa* LptD [46], and the antimicrobial peptide thanatin, which has been shown to interact with LptA and LptD *in vitro* and *in vivo* [47]. A recent study probed the *in vivo* Lpt network by utilising the bacterial adenylate cyclase two-hybrid approach and elegantly demonstrated that thanatin interferes with LptA-LptA and LptA-LptC interactions, being more effective towards the latter [48]. To further advance these new antibiotics, it is necessary to understand the LPS transport mechanism in detail. However, there remain several unresolved questions, for example, the substrate-induced conformational changes, the stoichiometry of the whole complex, and LPS transport and insertion mechanisms are still poorly understood.

Defining the interactions of heterogeneous LPS with multiple proteins, potentially in different conformational states, is a challenge for traditional structural biology approaches. Few methodologies used in isolation can capture the impact of the binding of lipids or antibiotics on the conformational states of heterogeneous membrane assemblies. Combinations of approaches are therefore required. Bringing together native MS, with hydrogen/deuterium exchange MS (HDX-MS) and molecular dynamics (MD) simulations, enabled details about substrate recognition and translocation to be revealed for LptD and the peptide antibiotic thanatin [49] (Figure 1b, 1c). LptDE was found to bind preferentially to LPS when compared to other membrane lipids such as phosphatidylglycerol (PG) and cardiolipin. Interestingly, thanatin did not compete with LPS for LptDE binding (figure 1b). HDX-MS analysis supported interaction of LPS with the periplasmic domain and implied that LPS binding causes opening of the β -taco cavity in LptD, consequently inducing concerted break of hydrogen bonds between β -sheets forming the domain, leading to strand separation (figure 1c). These results were supported by molecular dynamics simulations. In addition, HDX-MS indicated that LPS reduces the dynamics of strands in the transmembrane region adjacent to the LPS exit gate. This is consistent with conformational rearrangements of residues in this region that are necessary to accommodate substrates, further supported by MD simulations. Notably, LptE undergoes only subtle conformational changes, mostly related to substrate-induced altered dynamics of the LptD β -taco, with which LptE is in contact through these regions. In summary, using native MS, HDX and MD simulation the binding

of multiple LPS molecules to LptDE was observed elucidating a mechanism whereby one molecule at a time is passed through the exit gate.

The same approach indicated that thanatin binds at the N-terminus of LptD, similar to its LptA binding mode, and interferes with LPS-induced β -taco opening through stabilisation of the hydrogen bonding network between the β -strands, thus fixing this domain in a “locked” conformation. Remarkably, LPS is still able to decrease the dynamics of the β 1- β 4 strands, supporting the idea that concomitant binding of both ligands is possible. Thanatin however does not allow LPS sliding through the periplasmic portion [49]. Considering these studies, it appears that thanatin blocks LPS transport at multiple levels: as a disruptor of the interactions between the components of the periplasmic bridge [48] and also as a negative modulator of LPS translocation through LptD [49].

Drug transport through multidrug efflux pumps

The slow influx of drugs across the OM is further compounded by the presence of numerous efflux systems that pump antibiotics out of the cell before they reach their cellular targets. Drug efflux is currently considered as a major mechanism of drug resistance not only in bacterial infections but also in many cancers. Multidrug efflux transporters in bacteria are diverse, both structurally and functionally, and transport a wide spectrum of structurally diverse chemicals including antibiotics, toxins, detergents and lipids, thus playing essential roles in pathogenesis, virulence and biofilm formation [50]. Inhibition of efflux pumps is therefore an attractive strategy to reverse multidrug resistance which further can help to restore the efficiency of our current antibiotics to treat bacterial infections. Multidrug efflux pumps encoded on the bacterial genomes can be grouped into many families including ABC, RND, MFS, MATE, SMR, AbgT, and PACE families [32,51,52]. Here we focus on recent insights obtained by MS methods for the RND (resistance-nodulation-cell division) and PACE (proteobacterial antimicrobial compound efflux) family efflux systems.

A prototypical member of the RND family is the *E. coli* AcrAB-TolC system. This tripartite complex consists of an inner membrane protein belonging to the RND family (AcrB) an outer-membrane protein (TolC) and a periplasmic membrane fusion protein (AcrA) which connects the other two proteins. Recent structural analysis by cryo-EM and X-ray crystallography on AcrB with various antibiotics and efflux inhibitors, as well as the structure of the fully assembled AcrAB-TolC complex, have significantly enhanced our understanding of binding pockets, mechanism of substrate transport and the options for inhibition [31,53].

Recent advances have also revealed the impact of resistance mutations and their synergy with antibiotics and associated structural dynamics. For example, conformational changes in both AcrB^{WT} and clinically relevant mutation (AcrB^{G288D}) were monitored by HDX-MS upon binding to the antibiotic ciprofloxacin and the efflux pump inhibitor PA β N (Phenylalanine-Arginine β -Naphthylamide) [54] (Figure 2a, 2b). The study revealed that ciprofloxacin binding caused only subtle changes within AcrB^{WT}, mainly in the PN2 subdomain, the central cavity, R2 domain and 1 α -helix. Conversely, PA β N caused increased dynamics within the PN2 subdomain and reduced fluctuations within the connecting-loop and extensive parts of the PC1/PC2 cleft of the drug-binding pockets. These HDX results suggest that PA β N causes concerted restraint on AcrB dynamics, notably restricting drug-binding pockets, as a mode of action of the inhibitor (PA β N). Further examination of AcrB^{WT} in the presence of both ciprofloxacin and PA β N revealed similar differential HDX profiles for both AcrB^{WT}-PA β N and AcrB^{WT}-ciprofloxacin-PA β N, suggesting that the antibiotic and inhibitor may be able to simultaneously bind at different sites, a result confirmed by fluorescence polarisation and MD simulations [54].

In the case of the multidrug resistance mutation, G288D (AcrB^{G288D}), which was found to cause resistance to some drugs in *Salmonella* [55], an increase in HDX was observed for several peptides spanning the PN2 region and decreased HDX within the PC1/PC2 regions and the connecting-loop in the absence of no drugs (Figure 2b). The addition of PAβN resulted in a similar change in HDX for AcrB^{G288D} as observed for AcrB^{WT}, although a reduction in HDX for PC1 and R2 domains indicates that AcrB^{G288D}–PAβN possibly undergoes further restricted motion compared to AcrB^{WT}–PAβN. In summary, a consistent increase in HDX was observed within the PN2 region and decreased HDX of the connecting-loop in the case of the G288D substitution compared to the apo form and for all three substrate conditions tested (ciprofloxacin, PAβN, and ciprofloxacin–PAβN). Overall, these results suggest that the G288D mutation modifies the structural dynamics of the translocation pathway in a substrate-independent manner, which is likely contributing to its modified substrate efflux.

The PACE family represents one of the newly identified drug transporter families and the prototype is the Acel protein from *Acinetobacter baumannii*. Acel has been shown to confer resistance to chlorhexidine, mediated by an active efflux mechanism energized by an electrochemical gradient of protons [56]. Further studies on Acel homologs from other species have expanded the substrate range for PACE family proteins to synthetic biocides including benzalkonium, acriflavine, proflavine, and dequalinium [57]. Acel shares a similar size and predicted secondary structure to other members of the SMR family and has a conserved glutamate residue (E15) located in the middle of the first transmembrane helix. To probe structural/functional similarities with the SMR family and to investigate the mechanistic details of how the conserved glutamic acid residue couples efflux with the pH gradient, the response of Acel wild type and the E15Q mutant to chlorhexidine assault was monitored by native MS [58]. The data revealed a monomer-dimer equilibrium for the Acel protein. A significant increase in the dimer population was observed at high pH values (>7) and following the addition of cardiolipin and chlorhexidine (figure 2c). Interestingly, these differences were not observed for other cationic biocides, including benzalkonium, acriflavine, and proflavine, confirming the specificity of Acel towards chlorhexidine. For the E15Q mutant, a similar monomer-dimer equilibrium and chlorhexidine mediated dimer formation were observed but the pH-induced dimer formation was not observed. These results imply that an analogous mechanism to that of the SMR (small multidrug resistance) family is operative with protonation, deprotonation of the Acel transporter playing important roles in dimerization and efflux mechanisms. Overall, these results for Acel indicate several similarities between SMR and PACE families in terms of their monomer-dimer equilibrium and a critical glutamic acid residue. Furthermore, changes in the oligomerisation of the regulatory protein, AceR that controls the expression of Acel could also be linked to chlorhexidine assault. Collectively, these results demonstrate several possible avenues to explore with respect to developing inhibitors to stop the chlorhexidine efflux process. More recently, the substrate specificity of Acel has been extended to naturally occurring polyamines as the physiological substrates [59], however, further studies are needed to probe their interactions and conformational dynamics, which can advance future drug design.

Perspectives and outlook

The past two decades have seen many revolutions in structural biology, exemplified primarily by cryo-EM. In parallel has been the emergence of MS advancing our understanding of the structure, function and dynamics of proteins, particularly of membrane proteins. In this short review, we have summarised the recent progress made in lipid and drug transport in bacteria using native MS and HDX-MS with a special focus on LPS transport and drug efflux systems. These examples hopefully provide substantial encouragement for the design and development of novel compounds, similar to the newly identified antibiotic, darobactin for example [6], that selectively kills Gram-negative pathogens

(interaction of darobactin with its cellular target is shown in figure 2d), for combating multidrug-resistant bacterial infections and spawn further investigations in these areas.

More broadly, challenges remain for MS in capturing the surrounding lipids from native membrane environments. Although lipid binding is often readily captured by MS from micelles questions remain over the effect of detergents in modifying lipid binding. To overcome these effects researchers have adopted procedures developed in conjunction with other structural biology approaches for example nanodiscs [13] and liposomes [60]. Ejection from native-like membrane environments after forming vesicles, also first applied for EM [61], and later developed for use in conjunction with MS (sonicated lipid vesicles (SoLVE)) hold great promise for retaining membrane proteins in their native-like environments [62]. Initial results, showing retention of interactions for subcomplexes of tripartite pumps, provide proof-of-concept data for further optimisation and the challenge with antibiotics. Often, antibiotics are discovered through their ability to impact bacterial cell growth but their molecular targets are not known. In this regard, MS is ideally placed to uncover binding targets within a bacterial membrane environment through ejection directly following the challenge with antibiotics. Where targets are known, but current antibiotics have established resistance mechanisms, assessing the effects of modified versions of these antibiotics, on lipid transport for example, could lead to new derivatives that can then be rapidly repurposed.

Moving beyond bacterial systems, further improvements in the ability to perform tandem MS [63] will aid in defining precisely the chain length and head group chemistry of lipids associated with macromolecular complexes. As a stand-alone technique, the strength of MS lies in its power to add the vital small molecule details to the overall view of complex macromolecular machines, particularly those defined at high resolution by cryo-EM [64]. Together with the post-translational modification status (glycosylation, lipidation, phosphorylation), screening of small molecules to membrane protein targets provides new opportunities for drug discovery important not just for antibiotics, but also for the plethora of new antivirals motivated by the current pandemic.

Conflict of interest statement

Nothing declared

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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Figures and legends:

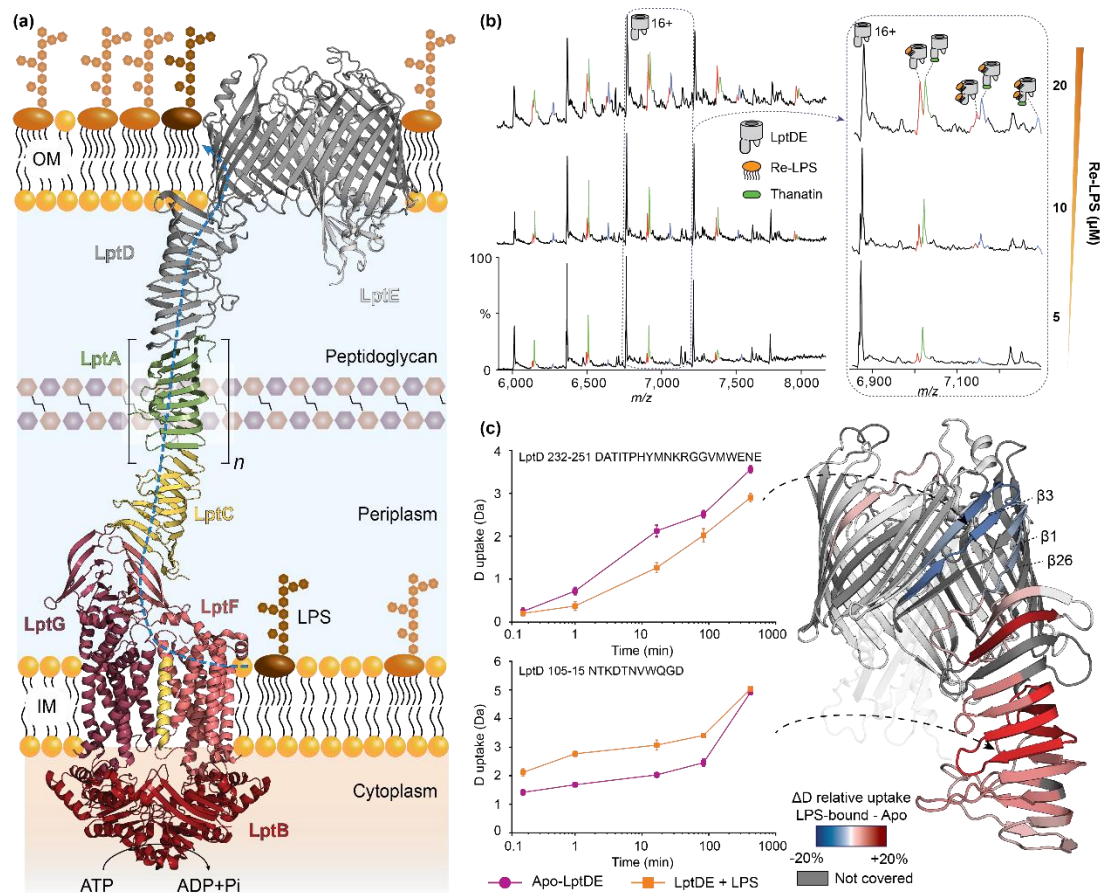


Figure 1: Schematic of the lipopolysaccharide (LPS) transport system. (a) LPS is transported from the inner membrane (IM) to the outer membrane (OM) through a multiprotein complex comprising seven proteins. LPS is extracted from the IM via LptB2FGC, and subsequently passes through periplasm via LptA oligomer, and eventually gets inserted into the outer leaflet of the outer membrane by LptDE. LptB2FGC utilize the energy from ATP hydrolysis to power LPS movement. (b) native MS analysis of LptDE with Re-LPS and thanatin. The charge state series corresponding to LptDE bound to Re-LPS (orange), thanatin (green) and both Re-LPS and thanatin (purple) are observed. (c) Conformational dynamics of LPS-bound LptDE obtained from HDX-MS. Top and bottom plots are the deuterium uptake of representative peptides (105–115, β -taco and 232–247, β -barrel) plotted as a function of labelling time (0.167–420 min) for apo-LptDE (purple circles) and LPS-bound LptDE (orange squares). The difference in relative deuterium uptake (scaled for the number of residues of each peptide) mapped on the crystal structure of LptD (PDB ID: 5IV9) is shown in the middle. Only peptides showing significant differences are coloured. Red and blue indicate increased and decreased deuterium uptake, respectively.

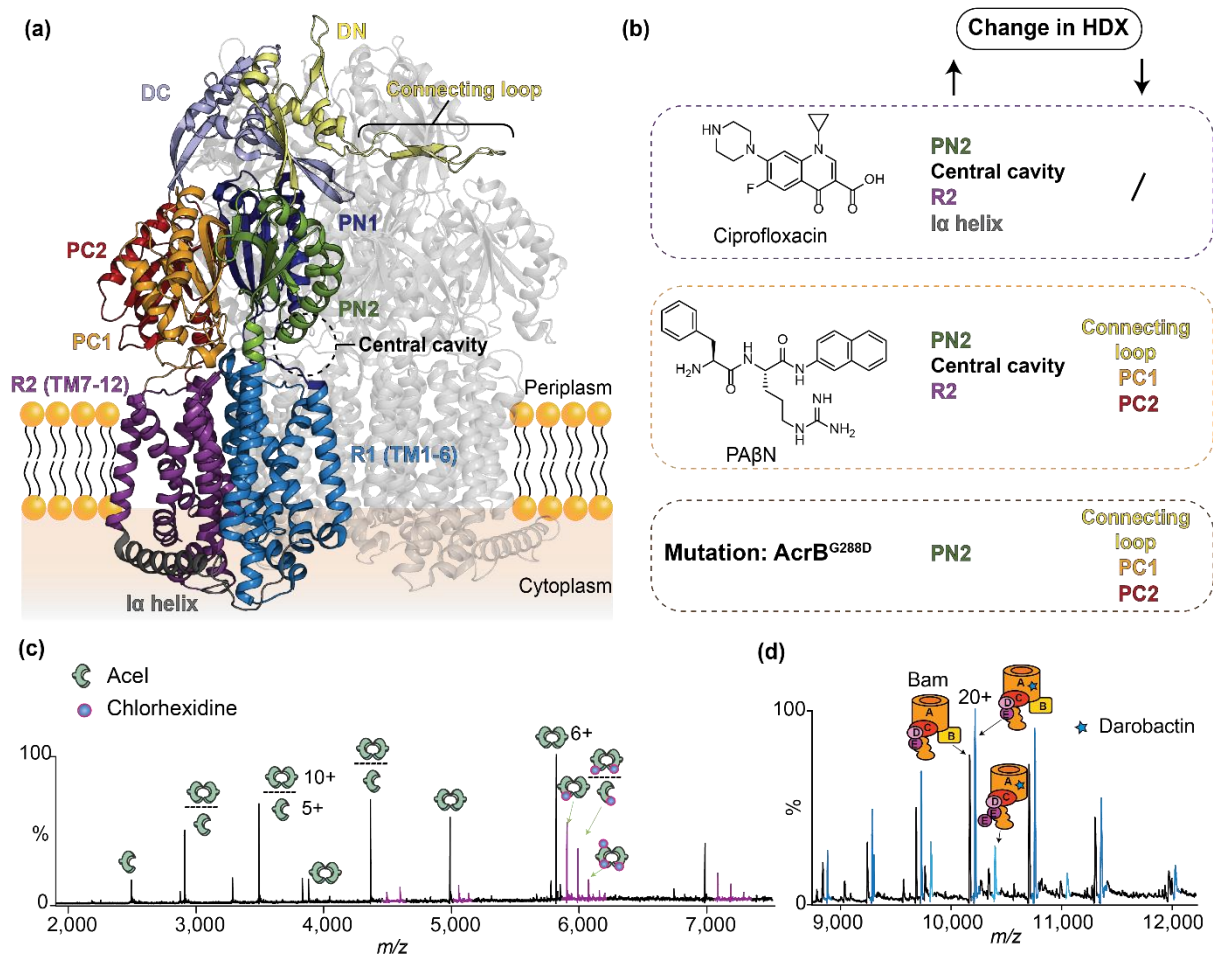


Figure 2: Antibiotic mediated conformational changes of drug efflux pumps. (a) Structure and subdomains of a representative member of the RND family, AcrB (PDB:2HRT). (b) Summary of changes in HDX observed upon addition of substrates (ciprofloxacin and PAβN) and for mutated AcrB (G288D). (c) Native mass spectra of Acel with chlorhexidine, note that the dimer peaks are only observed in the presence of the drug suggesting that chlorhexidine binding promotes dimer formation for efflux. Additional peaks adjacent to the main charge state distribution (pink) correspond to the mass of chlorhexidine. Multiple binding sites imply that there is not a specific binding site but rather an efflux of multiple drugs simultaneously. (d) Mass spectra of a newly identified antibiotic, darobactin bound to its cellular target, Bam complex, indicating that darobactin readily binds Bam complex and its sub complexes that exist in solution.