Abstract:

Understanding enzymatic catalytic cycles by quantitative crosslinking mass spectrometry and molecular simulations.

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The AcrAB-TolC pump is a proton-gradient powered drug efflux transporter capable of conferring resistance to a broad spectrum of antibiotics in *E. coli* and other Gram-negative bacteria. Despite the availability of several structures and computational studies of the AcrAB-TolC system, the proposed cycle of conformational changes involved in antibiotic specificity and drug efflux catalysis remains poorly understood. Here, we propose to tackle the mechanism of this system by integrating the acquisition and quantification of experimental distance restraint with molecular dynamics approaches. In order to derive the equilibrium between different conformers in solution, the Rappsilber laboratory will apply just developed quantitative crosslinking mass spectrometry (QCLMS) approaches in combination with novel photo-amino acid crosslinkers, capable of targeting transmembrane protein regions. These restraints will be used to aid molecular simulations of the system, performed in the Mroginski laboratory, as well as to model the “missing links” in the conformational cycle of the pump. The project will provide a full view of the dynamics involved in ligand binding and drug efflux catalysis by AcrAB-TolC.

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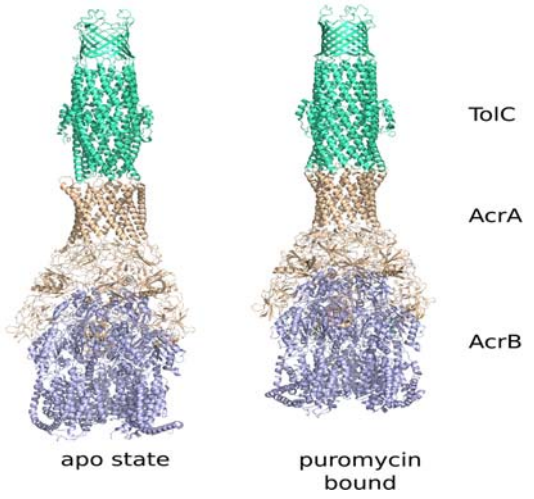
1. Overall goal of the project

We aim to unveil the mechanism of the AcrAB-TolC multidrug efflux pump system, which uses a proton gradient to catalyse the efflux of a broad spectrum of antibiotics classes in gram-negative bacteria. We will determine the conformation of the complex both in the free state and in the presence of several antibiotic types and under different conditions, to test and expand existing models of drug efflux catalysis and derive principles of ligand binding. We will collect distance restraint data by a variety of novel crosslinking mass spectrometry approaches, relying on photo-activatable amino acids, capable of targeting transmembrane regions both in purified proteins and in *E. coli* cells. Our restraints will combine with molecular modelling and molecular dynamics approaches to characterise the conformational changes involved in the drug efflux transport cycle of the system. Additionally, the experimental data will be used to gain insights into the broad substrate specificity of the pump, as well as to characterise the different pathways for drug extrusion across the membrane that have been previously observed.

2. State of the art



Biological background. The AcrAB-TolC system comprises the outer membrane channel TolC and the inner membrane transporter AcrB coupled by the intermembrane-spanning protein AcrA. Cryo-EM structures of the full enzyme have been solved in the resting and pumping state, as well as crystal structures of individual components in several conformational states. The combination of structural and computational analyses has led to the proposal of a rotary mechanism for the AcrB transporter, coupled to drug efflux via a peristaltic pump motion, and to several mechanisms for drug binding and efflux. Despite these efforts, the cycle of conformational changes involved in ligand binding and transport remains unclear.

Technical developments. Standard crosslinkers are water soluble, target hydrophilic amino acids and are fairly long, all making them non-ideal for work with membrane proteins and conformational changes. Since their introduction, photo-leucine and photo-methionine promised delivering short-range distance restraints in membrane proteins. However, photo-leucine is poorly incorporated (0.7%), and quantification approaches for crosslinks are generally lacking. Over recent years, our group has advanced in photo-amino acid crosslinking approaches in collaboration with Ned Budisa. For *E. coli*, we saw photo-leucine incorporate up to 30% of the native amino acid. Photo-methionine can even reach 50%. With such crosslinking densities, photo-amino acids can lead to valuable distance restraints, especially in hydrophobic proteins, which are leucine-rich.

The combination of quantitative crosslinking together with computational modelling approaches is a relatively novel approach capable of revealing conformational changes in a system. It relies on estimating changes in the abundance of crosslinks connecting residues in different conformations. Our lab has previously developed and used quantitative crosslinking together with a modelling approach based on the integrative modelling platform (IMP) to reveal the conformational changes in the structural complement protein C3 upon its activation and proteasomal lid maturation.

3. Specific aims and how they may be reached

Work package 1: CLMS characterisation of the AcrAB-TolC system in living cells.
Proposed work: We will establish production of the AcrAB-TolC system in *E. coli* according to established protocols, modified to include incorporation of photo-leucine and photo-methionine. We will then acquire photo-CLMS data on *E. coli* cells overexpressing the AcrAB-TolC system using our proteomics setup with state-of-the-art LC-coupled mass spectrometers (Lumos Orbitrap tribrid mass spectrometer and Q Exactive HF, Thermo). The protein complex will be characterised in the presence and absence of different antibiotic classes (macrolides, beta-lactam) to which AcrAB-TolC is known to confer resistance. The photo-amino acid data will provide contact (<8Å) information on the complex under different conditions.
Additionally, we will use established purification protocols to enrich for the protein complex *in vitro* longer-range (<30Å) CLMS using soluble crosslinkers, as previously established in our laboratory. This will help characterise the predicted large conformational changes (peristaltic motion, rotation) involved in coupling the proton gradient to the catalysis of drug extrusion.
Measure of success: Success will be defined by the acquisition of photo-amino acid crosslinks corresponding to various states of the AcrAB-TolC system in *E. coli* cells. The various states of the transporter will be further characterised by the acquisition of long-range restraints, capable of giving insights into rigid body motions and larger-scale conformational changes.

Work package 2: Quantification of conformational equilibria in solution.
Proposed work: We will apply state-of-the art quantification techniques to our *in vitro* crosslinking data acquired on the system in different states. The various distance abundances will then be combined with available structural knowledge to derive the abundance of different conformations of the complex (resting state, pumping state, protonated, deprotonated), under different conditions. This will enable us to derive a model for the equilibria between different previously known conformations involved in the transport cycle. If our experimental approach will lead to distances that are incompatible with existing models for the complex, we will use the distances to calculate alternative conformations of the enzyme by flexible docking or other molecular modelling approaches, as appropriate. By combining our *in vitro* crosslinking data with data acquired on the enzyme in different states, we will derive calculations of models at various stages of the pumping cycle, starting from known enzyme structures.
Measure of success: Success will be defined by a characterisation of the equilibrium between the individual states of AcrAB-TolC and the development of a simplified model for the catalytic transport cycle of the protein within the cell based on the CLMS data.

Work package 3: Characterisation of the structural dynamics and ligand binding of AcrAB-TolC.
Proposed work: Led by Prof. Mroginski, we will use the experimentally-derived distances to aid molecular dynamics simulations of the conformational changes involved in the catalytic cycle of the transporter. The large size of AcrAb-TolC combined with the complex membrane environment, constitute a major challenge for conventional modelling techniques. Therefore, computations will be performed considering a coarse grained molecular mechanical description of the entire system and modern enhanced sampling techniques combined with an efficient backward approach to convert the coarse-grained model to all-atom resolution. We will use our experimental data to aid computation in two main areas, namely in understanding the principles behind the unique ability of AcrAB-TolC to bind different classes of ligands, and in understanding the conformational dynamics involved in coupling the proton gradient to the transport of drugs via the multiple predicted channels.
Measure of success: Success will be defined by the development of methods to combine molecular dynamics with experimentally-derived CLMS restraints and the application of these novel methods to the characterisation of the conformational changes involved in drug transport and ligand binding.