Solid-state NMR: Insights into the Bacterial Autotransport Process

Structural studies of membrane proteins are challenging. Their heterogeneous membrane environment leads to purification problems. Membrane proteins are notoriously difficult to crystallize, in part because of their hydrophobic nature, and in part because of the presence of detergents from the purification procedures, while solution NMR spectroscopy is limited by the larger apparent size of the proteins that is added by the detergent micelles. Solid-state NMR is an emerging technology in membrane protein research that can overcome some of these problems, as it is sensitive to the chemical environment of individual molecules, but does not require large, well-ordered crystals. In our work, we have solved the structure of a transmembrane domain of a bacterial autotransporter, using solid-state NMR. For the first time, microcrystals were used to directly obtain a membrane protein structure at high resolution.

Bacterial Adhesion
Bacterial pathogens can enter their host organism by different routes: they can be taken up with food, can be inhaled, can enter through wounds, or can actively invade tissue through diverse mucous layers in the mouth, the intestine, or the urinary or genital tract. In all cases, the first step in the process of pathogenesis is bacterial adhesion, that allows the pathogen to stay at the site of infection long enough to multiply and to cause damage. Bacterial pathogens carry an arsenal of different adhesins for different specific purposes. These adhesins are in direct contact with the host environment, and interact directly with the immune system, which in some cases makes them interesting vaccine targets. But if bacterial adhesion could be inhibited, infestation of the host could be avoided from the start. Direct inhibitors of bacterial adhesion in everyday life are soaps and detergents that can disrupt adhesive interactions of bacteria with surfaces and with the skin, and on top of this can sometimes function directly as bacteriocidal compounds by lysing the bacterial cells. Modern antibiotics kill bacteria by membrane disruption, or by blocking protein synthesis, cell wall synthesis or DNA replication and packing. But in an era where resistance against such general inhibitors is spreading among
pathogens, new therapies are in high demand.

One obvious drug target is bacterial adhesion, either by blocking adhesion itself, or by inhibiting the biogenesis of the adhesive molecules on the surface of the pathogens.

**Autotransport**

Many different classes of molecules display adhesive functions in bacterial pathogens, ranging from carbohydrate structures over simple, fibrous proteins to complex oligomeric protein structures that extend far from the bacterial cell surface [1]. One class of such adhesins that is present in many important Gram-negative pathogens are the trimeric autotransporter adhesins (TAAs). The best-studied exemplar of this adhesin class is YadA [2] from the food-borne pathogen Yersinia enterocolitica. It is involved in initial adhesion of these bacteria in the gut, where they can cause severe diarrhea. In more complicated cases, *Yersinia enterocolitica* can invade deeply into the host tissue and cause chronic infections that lead e.g. to the formation of abscesses, or to reactive arthritis. YadA is essential for the colonization of the host; it allows the pathogen to adhere to collagen, a major surface component of the host cells. YadA is widely used as a model protein to study the biogenesis of TAAs. This biogenesis proceeds via a process that is conserved in many Gram-negative bacteria, called autotransport [3]. In short, the YadA protein chains are produced in the bacterial cytoplasm and are exported to the cell surface in multiple steps. In the last step, three YadA monomeric chains form a trimeric pore with their C-terminal ends, through which the rest of the molecules is exported through the outer membrane to the cell surface, where they fold and trimerize into the active adhesin. This autotransport process has no similarity to any known transport process in eukaryotes - which is an important prerequisite when developing small-molecule antimicrobial drugs, that ideally should not cross-react with host processes.
Solid-State NMR Technology

Structural studies of membrane proteins are challenging. Their heterogeneous environment leads to purification problems. Membrane proteins are notoriously difficult to crystallize. Many, if not most crystallization trials result in poorly diffracting crystals. The molecular weight of membrane proteins and difficulties in obtaining well-diffracting crystals hamper the application of the two most highly developed methods in structural biology: solution-state NMR and x-ray crystallography. Solid-state NMR (ssNMR) is a versatile alternative to study membrane proteins at atomic resolution in native, powder or crystalline states. ssNMR does not depend on the size or spatial arrangement of the biomolecules and is therefore special among high-resolution structure determination tools. In a technique called magic-angle spinning (MAS), a small sample container is spun at high frequencies (typically 10-40 kHz) around an axis inclined at the ‘magic angle’ of 54.7° relative to the magnetic field. As a result of this, the orientation-dependent dipolar coupling and chemical-shift anisotropic effects are averaged out. MAS NMR spectra show peaks that are sharp enough to facilitate the chemical shift assignment - a massive puzzle of correlating the observed spectral resonances to the backbone and side chain nuclei of the constituent amino acids.

To study the autotransport process of YadA, we produced a short construct that comprises only the membrane transport domain (called YadA-M), but not the adhesive part. We have used a microcrystalline preparation of the homo-trimeric membrane protein YadA-M from failed crystallization trials for structure determination by solid-state MAS NMR. We could successfully assign the full YadA-M sequence in a simple yet practical, step-wise fashion. Unique single residues and pairs of residues provided several potential starting points for unambiguous sequential assignment [4]. The program Talos+ [5] was used to predict an N-terminal alpha helix and four anti-parallel beta strands per monomer from the backbone $^{15}\text{N}$ and $^{13}\text{C}$ chemical shifts and the primary sequence of YadA-M. Medium- and long-range distance contacts were measured by tuning the polarization-exchange time in different mixing schemes [6]. In these correlation schemes, radio-frequency pulses are applied to reintroduce the structurally relevant dipolar couplings or promote diffusion-like exchange processes. The correlation peaks provide a direct measure of the three-dimensional fold of the protein. Selectively or sparsely isotope-labelled protein samples are routinely used in ssNMR. By this strategy, unambiguous distance restraints are obtained with much more convenience, albeit at higher costs of sample preparation. It is therefore important to note that the structure determination of YadA-M was entirely based on a single, uniformly $^{15}\text{N},^{13}\text{C}$-labelled preparation, and hence introduces a new paradigm for the field. Our work shows for the first time that a high-resolution...
ssNMR structure of a membrane protein can be solved from a single, uniformly isotope-labelled microcrystalline sample that was "discarded" for use in x-ray crystallography. The fact that we had only a single uniformly labelled sample to our disposal somewhat framed the choice of suitable NMR experiments for obtaining structural data; hence the NMR data set plays a crucial role in this respect. We used different experiments to extract structural information, for example, inter-strand contacts were predominantly established through ChhC and NhhC spectra. Filtered restraints, where one or both of the interacting carbon nuclei belong to flexible side-chain groups, were obtained by designing methyl-filtered correlation spectra. A detailed treatment of the used NMR experiments and their spectral information is given in the supplementary information of reference [7].

**Computational Challenges**

To compute an atomic resolution structure from solid-state NMR data is still a challenging task, mainly due to the ambiguity and imprecision of the distances. In case of YadA-M we had to fight the additional difficulty that distances can either refer to contacts within the same subunit of the homotrimer or contacts between interfacing subunits. These problems were tackled by developing a new structure calculation strategy. First a rough model of the subunit was computed which was later refined and assembled into a trimeric arrangement. To deal with the imprecision and ambiguity of the data we used a statistical structure calculation approach (ISD), which was shown to be particularly suited for challenging data sets because of its robustness and the statistical treatment of the data.

Using solid-state NMR we could solve a de novo structure of the membrane protein YadA-M (Fig. 1A). In addition to the structure, we identified a group of relatively flexible residues in the N-terminal coiled-coil region of YadA-M, which remained unnoticed in x-ray structure of the homologous protein Hia [8]. A stretch of alpha-helical residues - named ASSA region after the four involved residues - in YadA was identified as potential core for the formation of an intermediate hairpin during the autotransport. A drop in signal intensity, a set of non-helical chemical shifts and a lower predicted order parameter for the ASSA region were observed from NMR data. Small side-chain residues are present both on the interior-barrel wall and the part of coiled-coil that lies inside the beta barrel. Evolutionary studies also suggest that the low helix propensity, low hydrophobicity and small side-chain volume for ASSA region is conserved throughout the TAA family. We found several NMR interactions between the ASSA region and barrel residues, which help stabilize the structure after completion of the autotransport. The ssNMR data suggest that transport of bulkier head and stalk domains begins with a hairpin formation inside the beta barrel and confirm the "hairpin model" of autotransport in TAAs (Fig. 1B).
Outlook
The details on the structure and on the autotransport mechanism obtained from the solid-state NMR experiments can now be used to design more detailed experiments, with the long-term goal to design drugs that would block the process of TAA biogenesis. Such drugs could prevent the onset of infections and adhesion-dependent spreading of pathogens within the host by inhibiting bacterial adhesion.

References

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