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MsbA – an ABC Transporter Paradigm

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Abstract

ATP-binding cassette (ABC) transporters play an important role in various cellular processes. They display a similar architecture and share a mechanism which couples ATP hydrolysis to substrate transport. However, in the light of current data and recent experimental progress, this protein superfamily appears as multifaceted as their broad substrate range. Among the prokaryotic ABC transporters, MsbA can serve as a paradigm for research in this field. It is located in the inner membrane of Gram-negative bacteria and functions as a floppase for the lipopolysaccharide (LPS) pre-cursor core-LPS, which is involved in the biogenesis of the bacterial outer membrane. While MsbA shows high similarity to eukaryotic ABC transporters, its expression in Gram-negative bacteria makes it conveniently accessible for many experimental approaches from spectroscopy to 3D structure determination. As an essential protein for bacterial membrane integrity, MsbA has also become an attractive target for the development of novel antibiotics. Furthermore, it serves as a model for multidrug efflux pumps. Here we provide an overview over recent findings and their relevance to the field, highlight the potential of methods such as solid-state NMR and EPR spectroscopy and provide a perspective for future work.

Introduction

The superfamily of ABC transporters contains a variety of proteins from all domains of life that fulfil a multitude of functions in cellular processes. Defects in ABC transporters are connected to diseases such as cystic fibrosis[1], while fully functional ABC transporters can also pose a health risk for humans by acting as multidrug exporters with a broad substrate specificity in the context of bacterial antibiotic resistance or cancer chemotherapy (P-glycoprotein)[2]. ABC transporters utilize Adenosine triphosphate (ATP), bound and hydrolysed by the ATP-binding-cassette in the highly conserved cytosolic nucleotide binding domain (NBD) to facilitate transport. Transport itself takes place through the non-conserved transmembrane domain (TMD), in which several transmembrane helices (TMHs) form a pathway, which is highly specific to the type of substrates (proteins, peptides, sugars, lipids). A functional ABC transporter consists of two TMDs and two NBDs, which can be encoded separately or as fused domains. Other components such as substrate binding proteins, as in the case of importers, can be involved in the transport process. According to their TMD folds, ABC transporters can be classified into 7 different types[3]. In most cases, ATP binding or hydrolysis involving both NBDs allows the flexible TMDs to change their conformation between inward- and outward-facing states, which seems a key step in the transport cycle.

The progress in ABC transporter research is frequently discussed and reviewed, which is mainly due to the progress in 3D structure determination by X-ray and increasingly single particle cryo-EM (see for example [4-5]). Here, we restrict ourselves to MsbA (64 kDa) as a showcase example to demonstrate structural and mechanistic aspects as well as advanced biophysical methodology. MsbA is a type IV[3] half-transporter with one TMD and one NBD encoded on one polypeptide chain. As a homodimer, MsbA is located in the inner membrane (Figure 1) of various Gram-negative bacteria such as *Escherichia coli, Acenitobacter baumanii* or *Salmonella typhimurium*.[6-7] Originally discovered as a Multicopy suppressor of the HtrB temperature-sensitive phenotype that is essential for bacterial survival, sequence analysis revealed its connection to the ABC transporter superfamily.[6] First studies showed involvement of MsbA in lipid transport, identifying the translocation of core-LPS as its major function.[8] This endotoxin molecule consists of the lipid A membrane anchor and the inner and outer core oligosaccharides (Figure 1a). After MsbA mediated translocation of core-LPS, LPS is formed by addition of the O-antigen and transported to the outer leaflet of the outer membrane in Gram-negative bacteria.[9] Those molecules are essential to protect the bacterial cell and trigger the innate immune response during bacterial infections. In addition to core-LPS, glycerophosplipid transport is also partly depended on MsbA, which provides this protein with an outstanding role for bacterial cell membrane integrity.[10-12] Besides lipids, MsbA is also able to transport a wide range of small organic molecules such as dyes or drugs making it a model for ABC multidrug efflux pumps such as P-glycoprotein.[10]

As a prokaryotic protein, MsbA is experimentally better accessible than most eukaryotic ABC transporters, which has resulted in a wealth of biochemical, structural and kinetic data obtained by a multitude of methods such as for example luminescence spectroscopy[13], X-ray crystallography[14-16], single-particle cryo-EM[17-19], EPR[20-22]- and solid-state NMR spectroscopy[23-24]. These data revealed novel insights and provide a unique possibility towards a better mechanistic understanding of ABC transporters.

The 3D structures of MsbA

Getting high resolution structures of MsbA has been challenging. First X-ray crystallography data revealed the backbone conformation at lower resolution (PDB: 3B5W, 3B5X), but addition of nucleotides helped to stabilize the protein in specific states of the ATPase and coupled transport cycle (PDB: 3B5Y, 3B5Z, 3B60).[16] More recently, the structure of MsbA reconstituted into nanodiscs was solved by cryo-EM with a resolution of 4.2 Å (PDB: 5TV4).[18] Electron densities inside the MsbA dimer could be attributed to bound core-LPS and a stabilizing interaction network between protein and substrate could be identified. This study also reported a transition state structure with displaced TMH6/6' blocking the core-LPS-free binding pocket (PDB: 5TTP). These data led to the proposal of a trap-and-flip transport model for core-LPS transport by MsbA. With the discovery of quinoline class MsbA inhibitors, 2.9 Å structures with bound inhibitor and core-LPS molecules were reported by X-ray crystallography (PDB:

6BPL, 6BPP).[14] A further crystal structure of MsbA co-crystallized with lipid A revealed even more putative binding sites of the substrate, which could belong to the entry and exit pathways of core-LPS (PDB: 6BL6).[15] Another structure of MsbA with bound core-LPS (4.2 Å) was reported by cryo-EM in peptidiscs, a novel membrane mimic (PDB: 6UZ2, 6UZL).[17] Structures are summarized in Table 1.
It emerges from these data that MsbA samples a large conformational space during its catalytic cycle with an inward facing (IF) conformation in its apo state and an outward facing (OF) conformation when ATP analogues are used. These findings are in general agreement with data from other ABC transporters[4] and reflect an alternating access transport model in which the NBD-NBD distances change while the protein converts from the IF to the OF conformation. However, the IF state varies dramatically with respect to the observed NBD separations (see Table 1). A surprisingly wide-open conformation was observed when MsbA was crystallised[15-16] or probed by cryo-EM[19] or EPR spectroscopy[20] in detergent micelles. In contrast, other data, especially those with MsbA in nanodiscs, facial amphiphiles or peptidiscs show a smaller separation of the NBDs,[14, 17-18] However, most recently, also in nanodiscs a wide-open IF state was reported.[25] In general, a lipid environment (both in forms of nanodiscs and liposomes) seems favourable over detergent micelles, which could lead to unwanted effects.[26] Interestingly, lipids in nanodiscs show a higher degree of order compared to liposomes[27-28], which could influence the conformational equilibrium of embedded proteins. Further studies in a more native environment such as liposomes or even controls in cellular membranes would be needed to resolve this issue.

Transmembrane Domain

In contrast to the highly conserved NBDs, TMDs vary in sequence and structure since they are responsible for accommodating different substrates and facilitate the translocation process. MsbA consists of 12 transmembrane helices (6 of each monomer), which are intertwined. TMH1/2/3/6 of one monomer and TMH 4´/5´ of the other monomer form one wing of the TMD in the IF conformation. This organization is changed upon the formation of the OF state, in which one wing consists of TMH1/2/3´/4´/5´/6´.[16, 18] This interlocking is also found in Sav1866 and may contribute to the stability of the MsbA dimer.[29]
The core-LPS binding site is formed by the TMHs with a ring of hydrophilic amino acids coordinating the phosphorylated glucosamines, a hydrophobic pocket at the periplasmic site accommodating acyl chains of 12-14 carbon length and a more hydrophilic site facing the cytoplasm providing room for the core sugars (Figure 2a).[14, 18] This binding pocket is accessible to core-LPS via a lateral portal formed by TMH4/6 of each monomer and lined with positively charged amino acids. Recent structural data also hint towards a periplasmic core-LPS exit site formed by TMH1/2/3 upon OF-IF switching.[15]
In addition to core-LPS, also smaller molecules like drugs and dyes as well as phospholipids have been found as MsbA substrates, since they stimulate basal ATPase activity and were verified by binding, transport or growth assays.[10-12, 30-32] It was shown that non-competitive binding of lipid A can occur in addition to amphipathic drugs such as daunorubicin or Hoechst-33342.[31] Both molecules interact with TMH6, which contains a hotspot for change-in-specificity mutations (Figure 2a)[33], but daunorubicin also displays an effect on TMH4[34]. It is therefore possible that a larger drug binding pocket exists in MsbA with different overlapping binding sites for different substrates.
Besides of accommodating the substrate, translating the NBD-movements towards the periplasmic site for substrate release is a key task of the TMD. The TMD-NBD communication is facilitated by intracellular loops, also called coupling helices (CH) 1/2 (Figure 2b). CH1/2 are located at grooves on the NBD surface. Mutations reduce the ATPase activity and impair ATP binding. Molecular dynamic simulations of CH-mutants predict important non-covalent interactions between the CHs and NBD motifs.[35]
Another important structural feature is the tetrahelix bundle formed by TMH3/4 of each monomer (Figure 2a). This bundle at the intracellular extensions of the coupling helices is formed in the OF conformation and is in contact with the NBDs. Disruption of the polar interactions at the bundle interface reduces ATPase activity and also disrupts the ATP binding-dependent conformational change in the TMD region, while still maintaining substrate and nucleotide binding affinities[36]

Nucleotide Binding Domain

The NBDs of ABC transporters are highly conserved domains, which display a similar set of motifs, necessary for ATP binding and hydrolysis. MsbA displays all seven motifs (Walker A/B, Signature Motif, A/D/Q-loop, His-Switch) in its two NBDs, which can twist and dimerize in a head-to-tail manner in order to sandwich an ATP-molecule between Walker A/B, A/Q-loop and His-Switch of one NBD and the Signature motif and the D-loop of the other NBD (Figure 2c).[16] Sequence-wise the NBD-part of MsbA is 51% identical and 66% similar to P-glycoprotein.[6] Both NBDs are able to hydrolyse ATP in MsbA, although the question whether they are active in a concomitant manner is still open. Cysteine mutations of the amino acids involved in the formation of the conserved NBD motifs showed that not all conserved but only a select number of residues are essential to support functional ATPase activity and growth in MsbA.[22]

ATP-hydrolysis catalysed by the NBD and nucleotide binding are key events for the function of MsbA. However, it was also demonstrated for MsbA[23-24], other ABC transporters and for some soluble ABC proteins [37-40], that the NBDs are also able to catalyse a reverse adenylate kinase-like phosphoryl transfer reaction in which ADP is converted back to ATP and AMP. Such an activity could be of importance during situations of ATP depletion in the cell.[41] It was shown by solid-state NMR (see below) that this reaction involves the same conserved motifs within the NBD as needed for ATP hydrolysis but in addition transient nucleotide binding in between the NBDs involving the Q-loop takes place so that two ADP molecules can assume an appropriate binding mode for the phosphoryl transfer reaction to take place.[23-24]

Transport Mechanism

While many aspects of substrate translocation across the membrane by MsbA are still unexplored and the ATP:substrate stoichiometry remains unknown, the sum of current observations accumulates in the proposal of a “trap-and-flip” mechanism.[18] The core-LPS molecule is synthesized at the inner leaflet of the inner bacterial membrane and can enter the MsbA homodimer through lateral diffusion at an opening between TMH4/6 of each monomer, though the substrate selection mechanism still remains unexplained.[15-16] The NBDs of MsbA are either in a nucleotide-free or ADP-bound state, which allows the protein to sample a wide range of IF conformations, which are suitable to accommodate the large core-LPS substrate.[16, 19-20] Positively charged residues along the lateral gate may guide the core-LPS molecule towards the binding site.[15] At this position, a ring of hydrophilic, positively charged amino acids coordinates the negatively charged phosphates of the core-LPS molecule. The core sugar modifications are oriented towards the hydrophilic cytoplasmic cavity and the acyl chains position themselves inside the hydrophobic pocket[14, 18] The dynamic gate formed by TMH4/5 closes towards TMH2/3/6 and twists the NBDs. Nucleotide binding occurs at the NBDs by formation of an ATP sandwich between the Walker A and the signature motif.[16] Hereby the NBDs come together and dimerize, but only in the presence of Mg^{2+}.[22] ATP- and core-LPS binding are not ordered, as the affinity for ATP is not changed due to prior substrate binding. ATP-binding on the other hand reduces the affinity for core-LPS but not enough to release the molecule right away.[31] In this state, the conformational changes occurring at the NBDs due to dimerization are communicated to the TMD via the coupling helices and the tetrahelix bundle.[35-36] At this point, the core-LPS acyl chains may enter the membrane leaflet between TMH1/3. ATP-hydrolysis repositions TMH6, which blocks the substrate binding site and ejects the core-LPS molecule to the outer leaflet of the inner membrane possibly via a hydrophilic surface groove formed in the OF state.[15, 18] Inorganic phosphate is released from the NBDs and MsbA can revert to its IF conformation, while ADP can stay bound to the protein. Besides of these findings, MsbA-mediated transport has also been suggested to be aided by ΔpH.[42]

MsbA as a drug target – discovery of specific inhibitors

MsbA is potentially an attractive antibiotic target due to its central role in the maintenance of bacterial membrane integrity. Two classes of inhibitors have been reported so far. Quinoline-based inhibitors [14, 43] suppress ATP hydrolysis as well as core-LPS transport. In contrast, tetrahydrobenzothiophene (TBT)-based inhibitors were found to - surprisingly - stimulate ATP hydrolysis while disrupting transport. [44] X-ray crystallography revealed that the quinoline-based inhibitors bind in a pocket formed by TMH4/5/6 via the bulk membrane without competing with core-LPS (Figure 2a).[14] This study suggested inhibition as a two-fold mechanism: First, they lock MsbA in the IF conformation by an induced-fit binding connected with local unwinding of TMH4 and the formation of a new salt bridge. Second, an allosteric inhibition occurs by an asymmetric NBD displacement towards the membrane and disruption of the NBD – coupling helix interactions. In contrast, a recent cryo-EM study in detergent and nanodiscs describes that binding displaces the NBDs away from each other resulting in lower ATPase activity, which will lead to transport inhibition.[25] A different mechanism was suggested by the same study for the TBT-inhibitors: Upon TBT binding, MsbA switches from a wide-open to a collapsed IF state in which the NBD distances are drastically reduced. Such an observation could be consisted with the observed stimulated ATPase activity. TBT targets the main substrate core-LPS binding site resulting in a disturbed NBD-TMD communication via the coupling helices.
None of these inhibitors are already suitable for clinical tests[43-44], but their discovery demonstrates that known or new MsbA binding pockets are 'druggable' by small ligands. Therefore, structure-based drug discovery seems a promising route for developing novel antibiotics targeting MsbA. Since MsbA is not in the focus of already established antibiotics, existing resistance mechanism could be evaded. The key challenge will be to find compounds applicable for a clinical setting with suitable pharmacokinetic and pharmacodynamic properties.

Time-resolved methods, solid-state NMR and EPR spectroscopy

Solid-state NMR and EPR spectroscopy offer the possibility to obtain specific data needed to complement 3D structures in order to derive mechanistic models. Both approaches allow a high degree of flexibility with respect to the choice of the membrane mimicking environment. One strength especially of solid-state NMR based on magic angle sample spinning (MAS NMR) is the possibility to record well resolved NMR spectra of membrane proteins directly from liposome preparations as outlined below for MsbA.
^{31}P MAS NMR can be conveniently used to monitor nucleotide binding and turnover, since the ^{31}P isotope is NMR active and occurs at 100% natural abundance. The α-, β- and γP chemical shifts differ substantially between nucleotides and offer therefore a specific readout for the catalytic state in which MsbA is studied. The bound nucleotide population can be detected by ^{1}H-^{31}P cross polarisation (CP) experiments, which only shows signals of immobilized, i.e. bound nucleotides. An example is shown in Figure 3a. The successful generation of MsbA pre-hydrolysis (ADP.BeF_3) and transition (ADP.VO_4) states is monitored by the occurrence of the α- and β-P resonances. Simultaneously bound species such as AMP bound to MsbA-ADP.VO_4 can so be identified[23]. Subsequently, their interactions with surrounding residues can be probed by dipolar ^{31}P-^{13}C or ^{15}N-^{13}C correlation spectroscopy as illustrated here for ADP.BeF_3 and ADP.VO_4 bound in close proximity to the A-loop Tyr351 (Figure 3b). The observed cross peaks represent through-space interactions between nitrogens in ^{15}N-ADP.VO_4 / ^{15}N-ADP.BeF_3 and carbons in ^{13}C-labelled MsbA. These experiments were recorded with the help of dynamic nuclear polarisation (DNP), a hybrid method merging the sensitivity of EPR with the advantages of solid-state NMR spectroscopy. Furthermore, the observation of nucleotide turnover by real-time ^{31}P MAS NMR enables to simultaneously record progress curves for all involved nucleotide species (Figure 3c). In this way, the capacity of MsbA to catalyse not only ATP hydrolysis but also the possibility to convert ADP back into ATP and AMP was discovered[24], a phenomenon, which could be easily overlooked by conventional biochemical assays (see NBD section above). ^{15}N-^{13}C spectra of a labelled site in TMH6 (^{13}C-Ala314-^{15}N-Cys315) feature a relatively broad line shape in the apo-state with and without daunorubicin (Figure 3d). In the transition state, chemical shift changes and line narrowing are

detected. The NMR resonances becomes even narrower in the presence of substrate. In this way, changes in the local conformational space of specific sites within MsbA can be probed [34].

EPR spectroscopy has been extensively used by applying site-directed spin labelling to MsbA. Since MsbA is a homo dimer, introducing single cysteine mutations and spin labelling results in dipole-coupled spin pairs. Pulsed EPR experiments such as DEER/PELDOR enable then to probe distances between these spin labels in the range of up to 80 Å, which has been essential for probing the wide opening and closing of the NBDs during the catalytic cycle of MsbA[20, 45]. EPR also revealed that binding of ATP at the Walker A motif does not cause dimerization by itself, since major structural changes in the NBD only occur with added Mg^{2+} or after ADP*Vi trapping [22]. More recently, another – mutation- and label-free – approach has been used based on the substitution of the diamagnetic Mg^{2+} with paramagnetic Mn^{2+} (electron spin S=5/2). Replacing this essential metal ion does not affect the activity of MsbA but places a spin probe near the nucleotide binding sites. Pulsed EPR experiments such as RIDME allow then to determine Mn-Mn long-range distances directly via their dipole-dipole couplings [23] (see Figure 4a). Paramagnetic Mn^{2+} replacement even allows to go one step further and bridge the gap to NMR spectroscopy by probing the location of nearby nuclei. NMR-active nuclei (e.g. ^{13}C, ^{15}N, ^{31}P, ^{51}V) will be 'quenched' in a distance-dependent manner (PRE – paramagnetic relaxation enhancement) (see Figure 4b). The so obtained variations in peak intensities can be used to obtain Mn^{2+}-nuclei distances or simply for the purpose of supporting NMR resonance assignment by comparison with diamagnetic spectra (Mg^{2+}) as already shown for another ABC transporter[46]. Complementary, EPR hyperfine spectroscopy allows to detect those nuclei found in the direct environment of the metal ion, which would be completely quenched in the NMR spectra. In case of MsbA trapped in the catalytic transition state (ADP.VO_4), ^{31}P, ^{15}N, ^{51}V signals of nearby nuclei can so be observed (see Figure 4c). These spectra are usually recorded at lower magnetic fields and without the help of magic angle sample spinning which limits the resolution to nuclei types. This approach is however potentially very powerful for probing interactions within the catalytic centre involving the essential metal ion [21]. Even nucleotide turnover in the NBD within the detection sphere of Mn^{2+} can be observed by utilizing time-resolved freeze quenching (Figure 4d).

EPR and solid-state NMR spectroscopy, X-ray and cryo-EM report on conformational changes by trapping intermediate/transition states or even under turnover conditions (as shown for example by cryo-EM for TmrAB[47]), but addressing the kinetics associated with these changes in real time is highly challenging. Attempts to address this problem for MsbA by other methods were undertaken by time-resolved luminescence resonance energy transfer (LRET) under continuous hydrolysis conditions[48], single molecule FRET[49] and time-resolved FTIR on isolated NBDs[50]. Recently, a kinetic analysis of conformational changes became also possible by stopped-flow time-resolved small-angle X-ray scattering on MsbA in nanodiscs[51]. These data show that NBDs dimerize upon ATP binding with a significantly longer time constant compared to their dissociation upon hydrolysis. For all of these studies, the choice of experimental conditions such as type of the membrane mimic and temperature are extremely important.

Perspectives

- MsbA is found at the intersection between key questions of the ABC transporter field due to its function as core-LPS floppase, as potential antibiotic drug target and because of the wealth of available data, which turned it into a model ABC multidrug efflux pump. MsbA has also been the driving force for the application of new methods in the ABC transporter field.
- MsbA undergoes a connected catalytic and transport cycle which involves substantial structural changes between inward and outward facing conformations, by which core-LPS is translocated via a 'trap-and-flip' mechanism. ATP binding seems to lead to NBD dimerization, while ATP hydrolysis and/or inorganic phosphate release reset MsbA. It is unknown whether both NBDs bind/hydrolyse ATP simultaneously or sequentially and how many ATPs are required per translocated substrate. The ability of MsbA to catalyse also a reverse adenylate kinase reaction could provide a mechanism to adjust its activity to alterations in local nucleotide concentrations.

- Given the inherent conformational flexibility of ABC transporters, and MsbA in particular, structural evaluation in various membrane environments is warranted as some of them may preferentially stabilize distinct transporter states. Such matrices may include detergent micelles, nanodiscs or other membrane mimetics. Liposomes appear closest to a more native state and also experiments within cellular membranes should be considered. Time-resolved experiments either based on flash freeze-quenching or in real time will be important to understand the sequence of events in the catalytic/transport cycle. Methods such as vibrational spectroscopy, single molecule fluorescence, EPR – and solid-state NMR spectroscopy will play an important role to close gaps between 3D structures based on X-ray crystallography, cryo-EM and functional data.

Conflicts of interest

The authors declare no conflict of interests.

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Author contribution

Both authors wrote the paper and contributed to the figures.

Figures, Tables and Legends

Table 1: Known 3D structures of MsbA

PDB	Reference	Organism	Conformational state and NBD separation *	Membrane mimic	Method	Resolution	Ligand
3B5Y	Ward et al. (2007)[16]	*S. typhimurium*	Outward-Facing 25.1 Å	Maltoside Detergent	X-Ray	4.5 Å	AMP-PNP
3B5Z			Outward-Facing 25.5 Å			4.2 Å	ADP-Vanadate
3B60			Outward-Facing 25.6 Å			3.7 Å	AMP-PNP
3B5X		*V. cholerae*	Inward-Facing 43.6 Å			5.5 Å	Apo
3B5W		*E. coli*	Inward-Facing 80.7 Å			5.3 Å	Apo
5TTP	Mi et al. (2017)[18]	*E. coli*	Occluded 26.4 Å	Nanodisc	single particle cryo-EM	4.8 Å	ADP-Vanadate
5TV4			Inward-Facing 38.2 Å LPS inside			4.2 Å	Apo
6BPL	Ho et al. (2018)[14]	*E. coli*	Inward-Facing 34.3 Å LPS inside, Inhibitor bound	Facial amphiphile	X-Ray	2.908 Å	G907 Inhibitor
6BPP			Inward-Facing 33.4 Å LPS inside, Inhibitor bound			2.92 Å	G092 Inhibitor
6O30	Padayatti et al. (2019)[15]	*S. typhimurium*	Inward-Facing 77.1 Å	Facial amphiphile	X-Ray	4.47 Å	Apo
6BL6			Inward-Facing 76.3 Å LPS inside/on the surface			2.8 Å	Lipid A
6UZ2	Angiulli et al. (2020)[17]	*E. coli*	Inward-Facing 39.8 Å LPS inside	Peptidisc	single particle cryo-EM	4.2 Å	Apo
6UZL			Inward-Facing 37.6 Å LPS inside			4.4 Å	Apo

(*) NBD separation corresponds to the distance between the two NBD centers of mass

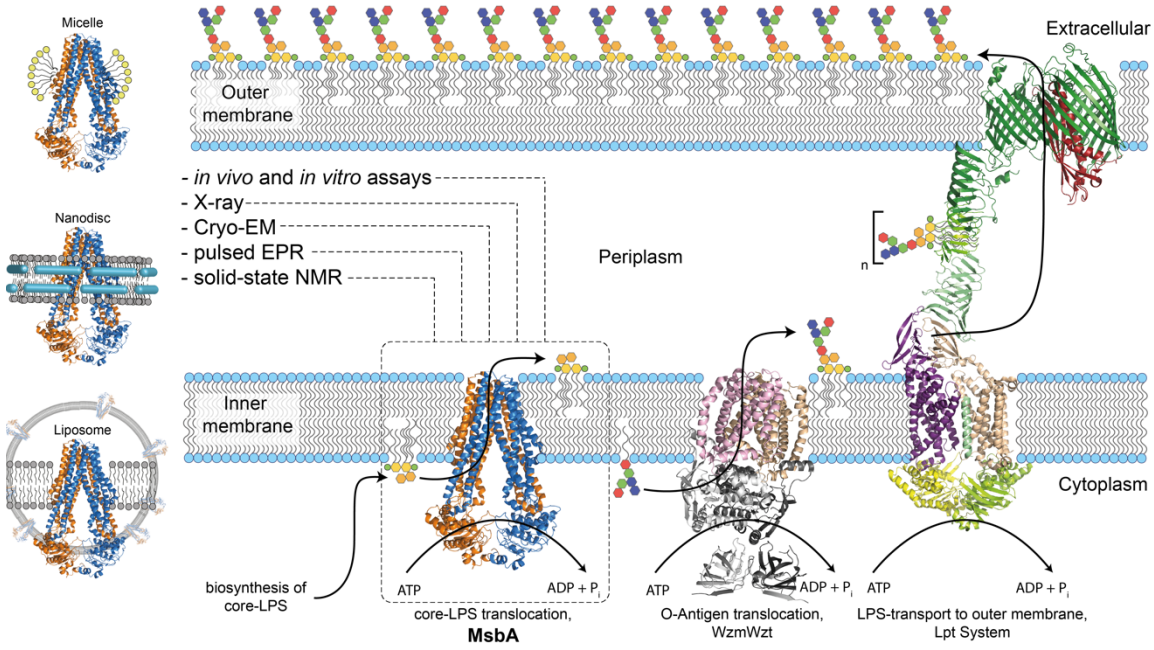

Figure 1: MsbA and lipopolysaccharide transport. Overview of the synthesis and transport steps of the outer membrane LPS components in Gram-negative bacteria. Lipid A is synthesized and modified with the core sugars (core-LPS) at the cytoplasmic site of the inner membrane and flipped by the ABC transporter MsbA (shown here PDB 6UZ2[17]). In the periplasm, the core-LPS is ligated to the O-Antigen, which is transported across the inner membrane by WzmWzt (PDB 7K2T[52]). The completed LPS molecule is transported to the outer leaflet of the outer membrane by the Lpt-system (PDBs: 6MIT[53], 2R1A[54], 5IV9[55]). MsbA is at the heart of this process and has been studied by a set of advanced biophysical methods. Various membrane mimics (left) from detergent micelles via lipid nanodiscs to liposomes have been used.

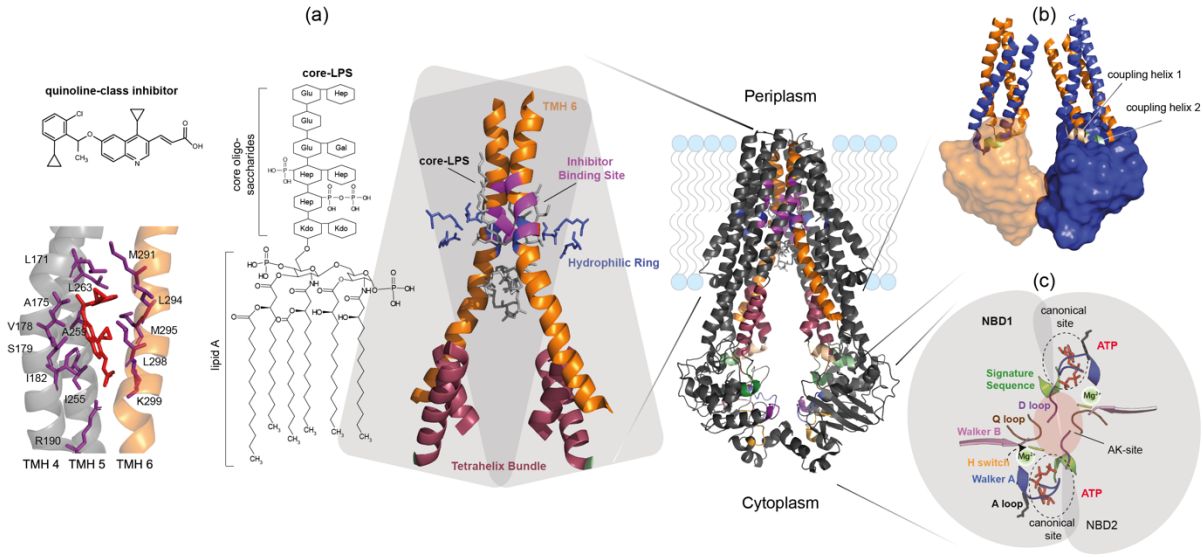

Figure 2: Structural motifs of MsbA. (a) TMD key elements (right) and ligands (middle, left). The core-LPS molecule (middle) is coordinated in the binding pocket by a ring of hydrophilic amino acids. TMH6, which plays a role in protein-substrate interaction also helps to form the binding site for quinoline inhibitors (left).[18] The cytoplasmic extensions of TMH3/4 are involved in the formation of a tetrahelix bundle.[36] **(b)** Contact between TMD and NBD. Coupling helix 1 and coupling helix 2 are shown with their TMD extension and contact sites at NBDs of both monomers. **(c)** NBD key motifs highlighted in the NBD dimer. The canonical binding site of the nucleotide is located between the signature sequence and the Walker A motif of the opposing NBD. Mg^{2+} ion coordination and hydrolysis are supported by the Q-loop, D-loop, Walker B motif, A-loop and His-Switch. Beside of ATP hydrolysis, MsbA has also been shown to catalyse a reverse adenylate kinase reaction, which involves transient nucleotide binding near the Q loop ('AK-site').[23] The cartoons shown here are based on structures PDB 5TV4 and 6BPL.[14, 18]

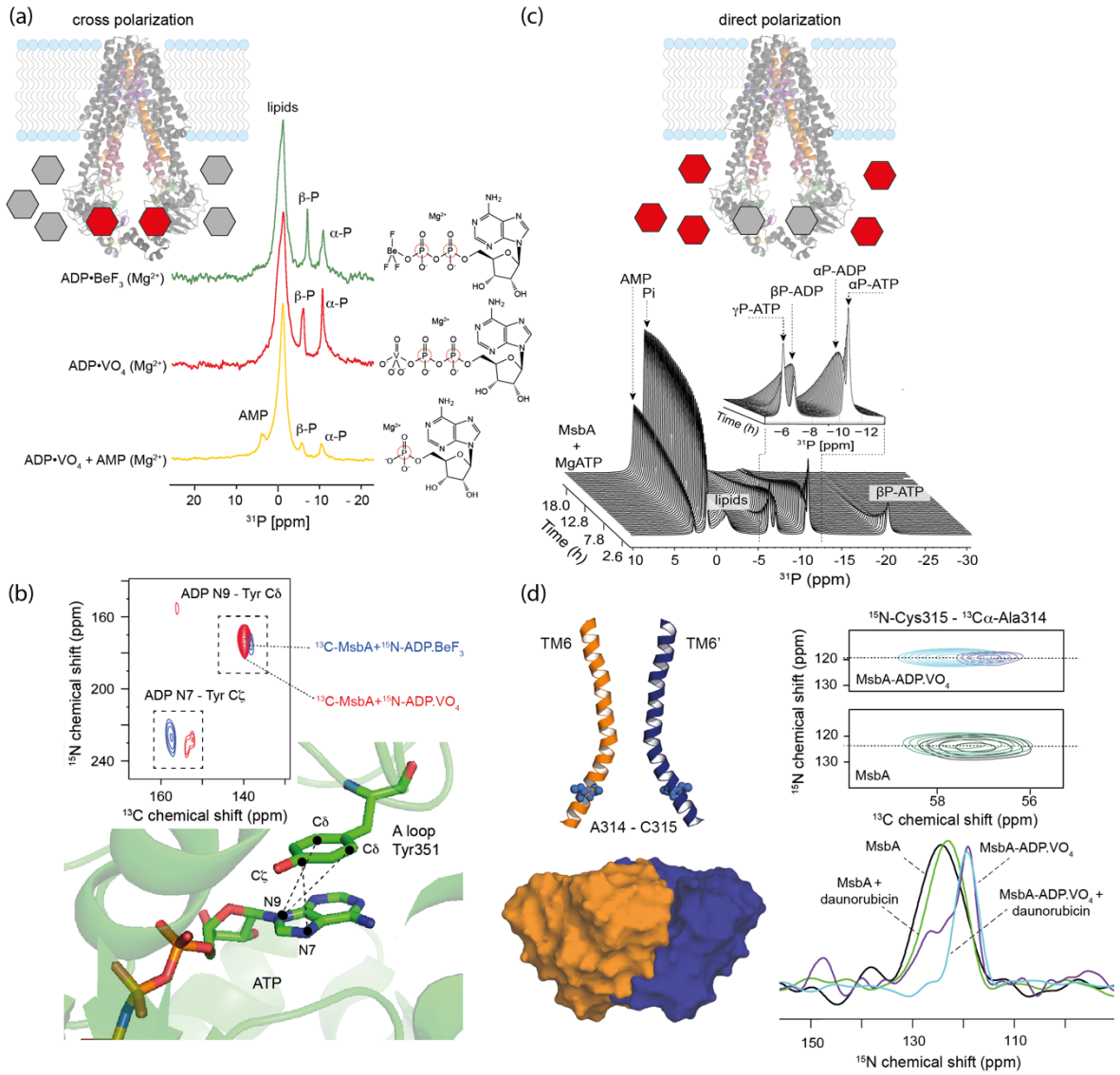

Figure 3: Solid-state NMR spectroscopy on MsbA proteoliposomes. (a) Detection of bound nucleotide by ^{1}H-^{31}P cross polarisation (pre-hydrolysis state MsbA.ADP.BeF_3, transition state MsbA.ADP.VO_4). The NMR experiment visualises only bound nucleotide species so that also new binding modes can be detected such as AMP bound along with ADP.VO_4 (MsbA.ADP.VO_4+AMP).[24] **(b)** The interaction between bound nucleotides and surrounding residues can be probed by DNP-enhanced through-space dipolar correlation spectroscopy as shown here for ^{13}C-MsbA and ^{15}N-ADP.BeF_3/ADP.VO_4. Specific crosspeaks with A-loop Tyr351 are observed.[23] The figure has been adapted from Kaur et al.[23] copyright 2018 with permission from the American Chemical Society. **(c)** Direct ^{31}P polarisation enables to monitor nucleotide turnover in real time. Here, progress curves for all species are recorded simultaneously. In this way, also side reactions which would be easily missed in biochemical assays can be detected, such as a reverse adenylate kinase reaction in which ADP is converted back into ATP and AMP.[24] **(d)** Substrate- and nucleotide induced conformational changes and plasticity within the TMD can be detected from chemical shift and line shape changes as shown here for a site in TMH6 (MsbA.ADP.VO_4+daunorubicin).[34] The figure has been adapted with permission from Spadaccini et al.[23], copyright 2018 with permission from Elsevier.

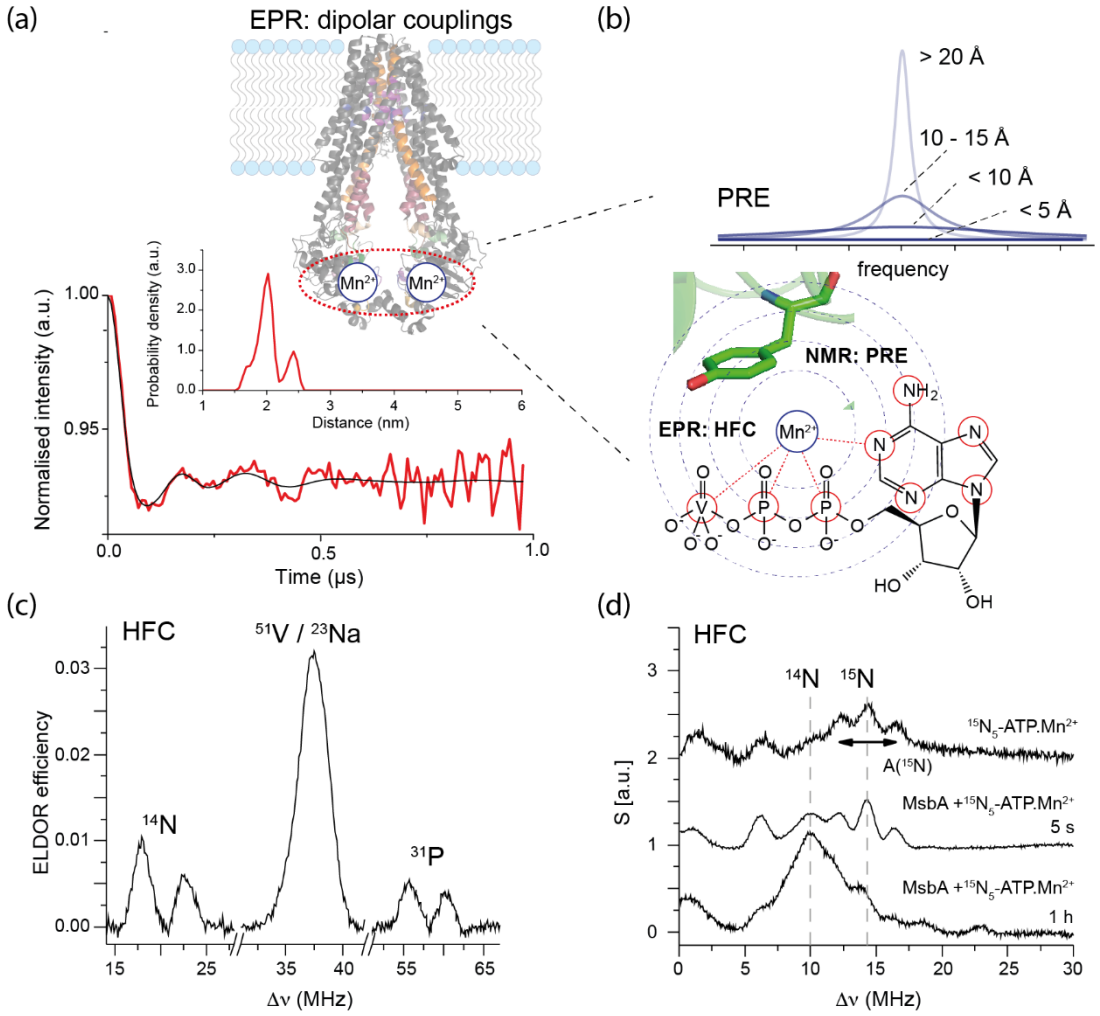

Figure 4: Utilizing Mn^{2+} for EPR (and NMR) spectroscopy on MsbA. (a) Mg^{2+} can be replaced by paramagnetic Mn^{2+} without affecting the activity of MsbA. Pulsed EPR experiments such as RIDME allow the determination of Mn-Mn long-range distances via their dipole-dipole couplings without the need of site-directed spin labelling.[23] The figure has been adapted from Kaur et al.[23], copyright 2018 with permission from the American Chemical Society. **(b)** The coupling between bound Mn^{2+} and adjacent nuclei within bound nucleotides or residues is visible in NMR experiments via distance-dependent signal loss through paramagnetic relaxation enhancement (PRE) or in EPR via hyperfine couplings as electron-detected NMR (EDNMR) spectrum. **(c)** EDNMR spectrum of MsbA.ADP.VO_4 shows ^{14}N, ^{31}P and ^{51}V nuclei in direct vicinity of Mn^{2+}.[21] **(d)** ATP turnover detected via Mn^{2+}-EDNMR. The ^{15}N signal of bound $^{15}N_5$-ATP observed after 5s incubation followed by freeze-quenching gradually decays with time [21]. The increasing ^{14}N signal arises from a residue carrying a nitrogen in close proximity to Mn^{2+}. Figures (c) and (d) were adopted from [21] and kindly provided by the authors. Figures (c) and (d) have been adapted from Collauto et al. [21], copyright 2017 with permission from Elsevier.

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