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# Effects of nucleotide binding to LmrA: A combined MAS-NMR and solution NMR study

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ABSTRACT

ABC transporters are fascinating examples of fine-tuned molecular machines that use the energy from ATP hydrolysis to translocate a multitude of substrates across biological membranes. While structural details have emerged on many members of this large protein superfamily, a number of functional details are still under debate. High resolution structures yield valuable insights into protein function, but it is the combination of structural, functional and dynamic insights that facilitates a complete understanding of the workings of their complex molecular mechanisms. NMR is a technique well-suited to investigate proteins in atomic resolution while taking their dynamic properties into account. It thus nicely complements other structural techniques, such as X-ray crystallography, that have contributed high-resolution data to the architectural understanding of ABC transporters. Here, we describe the heterologous expression of LmrA, an ABC exporter from *Lactococcus lactis*, in *Escherichia coli*. This allows for more flexible isotope labeling for nuclear magnetic resonance (NMR) studies and the easy study of LmrA's multidrug resistance phenotype. We use a combination of solid-state magic angle spinning (MAS) on the reconstituted transporter and solution NMR on its isolated nucleotide binding domain to investigate consequences of nucleotide binding to LmrA. We find that nucleotide binding affects the protein globally, but that NMR is also able to pinpoint local dynamic effects to specific residues, such as the Walker A motif's conserved lysine residue.



## 1. Introduction

ATP Binding Cassette (ABC) transporters are involved in the transport of a multitude of different compounds including ions, nutrients, antibiotics and even peptides and lipids across cellular membranes [1]. ABC transporters recognize and translocate their substrates through a helical transmembrane domain (TMD). Their name stems from the use of ATP as their energy source to facilitate substrate transport. ATP is hydrolyzed in the so-called nucleotide binding domains (NBDs). The general architecture of ABC transporters features a structural dimer of two transmembrane regions and two nucleotide binding domains. An intriguing subset of ABC transporters does not move a single, defined substrate or substrate class across membranes but rather a large number of chemically unrelated compounds. These are the multidrug resistance (MDR) ABC transporters [2]. They play important roles in bacterial antibiotic resistance and resistance to chemotherapeutics in cancer treatment. LmrA, a bacterial homodimeric multidrug ABC transporter from *Lactococcus lactis* homologous to human P-glycoprotein is one such example [3].

The general features of substrate transport and ATP hydrolysis by ABC transporters have been elucidated by a vast number of structural and functional studies, but even so, a number of key concepts remain unclear. Especially in the realm of MDR transporters, details of how these proteins select and recognize their diverse substrates remain amiss. The coupling of ATP hydrolysis and substrate transport which goes hand in hand with large global conformational changes in the transmembrane domain is also not fully understood. Intriguingly, a

*Abbreviations:* ABC, ATP binding cassette; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CP, cross polarization; DDM, n-dodecyl-β-D-maltoside; EM, electron microscopy; EPR, electron paramagnetic resonance; HSQC, heteronuclear single quantum coherence; INEPT, insensitive nuclei enhanced by polarization transfer; ISOV, inside out vesicle; MAS, magic angle spinning; MDR, multidrug resistance; NBD, nucleotide binding domain; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); RSOV, right side out vesicle; TMD, transmembrane domain; TROSY, transverse relaxation optimized spectroscopy.

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number of studies on MDR ABC transporters indicated that they seem to be highly flexible, at least at certain intermediate points throughout their ATP hydrolysis and substrate translocation cycle [4–6]. Intrinsic flexibility is a feature they share with other multidrug translocators, such as members of the small multidrug resistance (SMR) protein family or the Resistance Nodulation cell Division (RND) superfamily [7–9].

For LmrA, it was shown previously by electron paramagnetic resonance (EPR) spectroscopy that there are vast changes in global dynamics between nucleotide-free and nucleotide-bound states [10]. Similar effects were observed for other ABC transporters e.g. [11]. It may be speculated that in addition to the overall global effects observed upon nucleotide binding, local dynamics will change as well. ABC transporters contain a number of highly conserved sequence motifs that are essential for domain communication and ATP hydrolysis [12,13]. We hypothesized that changes in protein dynamics should be especially apparent in these regions as they govern and collate information from substrate and nucleotide binding to fine-tune the concerted large range motions required for an ABC transporter to efficiently fulfill its task of moving substrates across the membrane.

In particular, catalytic residues within the NBD should probably be strongly affected. Membership in the ABC superfamily is contingent on the presence of the signature motif, the "C-loop". Other conserved regions within the NBD essential for ATP hydrolysis are the Walker A and Walker B motif. The Walker A motif has the consensus sequence G/AxxxxGKT/S (with x = any amino acid) [14]. The side-chain of the conserved lysine residue (K388 in LmrA) interacts with the γ-phosphate of ATP during the hydrolysis reaction. Its mutagenesis can lead to significantly decreased ATPase activity and/or disrupts nucleotide binding [15–18].

When an ABC transporter undergoes its substrate translocation/ATP hydrolysis cycle, ATP binding and/or hydrolysis at the NBDs is coupled to conformational changes within the transmembrane region. Both the substrate and the ATP hydrolysis products (free phosphate and ADP) are released and the transporter is reset for a new cycle. To stably "trap" intermediates of the ABC transporter cycle for spectroscopic investigations, pre-hydrolysis or transition state analogues are commonly used. Trapping is achieved after a single round of ATP turnover. The resulting ADP is trapped by the exchange of $P_i$ to a metal phosphate analogue (e.g. beryllium fluoride ($BeF_x$) or vanadate ($V_i$)) with a significantly higher affinity to the phosphate binding site. While beryllium fluoride mimicks the tetrahedral geometry of the pre-hydrolysis state, vanadate emulates the pentavalent bipyramidal architecture of the transition state. In ABC transporters, trapping with phosphate analogues was successfully demonstrated on numerous systems [19–21].

The tremendous improvements in crystallization-based structure determinations of secondary and primary active transporters create a need and offer the possibility for extensive spectroscopic studies to link structure and dynamic data with functional mechanisms. NMR is especially well suited since site-resolved data can be obtained for various catalytic states of proteins. In case of membrane proteins, solid-state NMR, in particular based on magic angle sample spinning, is the method of choice due to the possibility to work in the lipid bilayer [22–24].

Indeed, LmrA was the first ABC transporter for which the application of solid-state NMR has been attempted. These early trials focused first on establishing isotope labeling in *L. lactis* [25], on the application of wide-line $^2H$ NMR to probe the overall dynamics of LmrA [26] and on assessing the activity of reconstituted LmrA by $^{31}P$-MAS NMR [17]. Unfortunately, the limitations of the *L. lactis* expression system with respect to isotope labeling had limited further studies at that time.

It has been demonstrated in the meantime that promising MAS-NMR spectra can be obtained from ABC transporters expressed in *Escherichia coli*. Examples involve the thermophilic import system ArtMP [27], the *Bacillus subtilus* ABC exporter BmrA [28] as well as *E. coli* MsbA, which was probed in its apo-state as well as in complex with lipid A [29].

Here, we now report also for LmrA the heterologous expression in *E. coli* minimal and defined medium for isotope labeling for NMR studies. Our data show that LmrA from *E. coli* shows the same functional features as *L. lactis* LmrA which make testing of the integrity our NMR constructs very convenient and straight-forward. In *L. lactis* however, LmrCD has also been described as an important in vivo ABC MDR pump [30]. Sufficient amounts of isotope-labeled LmrA for NMR spectroscopy can be obtained. For solid-state MAS-NMR studies, the transporter was reconstituted into lipid bilayers. Its global dynamics was empirically assessed by temperature-dependent MAS-NMR detection through INEPT, cross-polarization and direct polarization experiments. Residue selective nitrogen labeling was used to probe the effect of nucleotide binding onto the spectral lineshapes of lysine and tryptophan residues. In addition, isolated isotope-labeled NBDs were prepared and the influence of nucleotide binding was probed by solution-state NMR.

## 2. Material and methods

All chemicals were purchased from Applichem (Darmstadt, Germany) unless indicated otherwise. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

### 2.1. Cloning of the lmrA gene

The *lmrA* gene was excised from the lactococcal plasmid pNHLmrA as a NcoI-SacI fragment and subcloned into the *E. coli* vector pET28b(+) (Novagen). The resulting plasmids were transformed into *E. coli* strain BW25113ΔAcrB (a gift from K.M. Pos, Frankfurt) for functional characterization or T7 Express cells (NEB) for labeling and expression for NMR experiments. As controls, empty pET28b or pET24acrB plasmids were used.

### 2.2. Protein expression and purification

For full-length LmrA expression, *E. coli* cells were grown at 37 °C in LB medium (Roth) with 25 μg/mL kanamycin sulfate at 205 rpm in an orbital shaker to an $OD_{660}$ of 0.8. They were then induced with 1 mM IPTG (Roth) and grown for another 2 h. LmrA–NBD was expressed and purified as previously described [31]. For isotope labeling defined medium supplemented with $^{15}N$-lysine or $^{15}N$-tryptophan (Eurisotope) was used. His-tagged full-length LmrA was purified from ISOVs and trapped as described [10]. Pure protein yields were determined with the DC Protein Assay (BioRad) and verified with SDS-PAGE. Total membrane protein and protein yield after purification were determined with the colourimetric DC Protein Assay (BioRad) and photometrically detected (Jasco V-550 UV/VIS Spectrophotometer).

### 2.3. Vesicle preparation

Inside–out vesicles were prepared by dissolving cells in 5 mL lysis buffer (250 mM sucrose, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5, 2.5 mM $MgSO_4$, 25 μg/mL DNaseI and protease inhibitors) per gram of wet weight cells. Vesicles were formed by three passes through a cell disruptor (Constant Systems) at 1.7 kbar. 15 mM K-EDTA, pH 8 was added to the lysate and cell debris removed by centrifugation. ISOV were then collected by ultracentrifugation and homogenized in 50 mM KPi buffer, pH 7, 10% glycerol. RSOV were prepared as previously described [32]. In short, cells were harvested in mid-exponential phase and lysed with 20 μg/mL lysozyme. Spheroplasts were harvested by centrifugation and then diluted 300-fold. Whole cells and spheroplasts were separated from membrane vesicles in a subsequent centrifugation step (60 min, 10,000 *g*). The pellet was homogenized and the suspension centrifuged. The supernatant was collected and centrifuged (30 min, 50,000 *g*) to harvest right side out vesicles. RSOV were aliquoted and stored in liquid nitrogen until use.