The transmembrane domains of the ABC multidrug transporter LmrA form a cytoplasmic exposed, aqueous chamber within the membrane

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\textbf{Running title}: Identification of solvent accessible residues in LmrA
SUMMARY

The ABC multidrug transporter LmrA of *Lactococcus lactis* consists of 6 putative transmembrane segments (TMSs) and a nucleotide binding domain. LmrA functions as a homodimer in which the two membrane domains form the solute translocation path across the membrane. To obtain structural information of LmrA a cysteine scanning accessibility approach was used. Cysteines were introduced in the cysteine-less wild-type LmrA in each hydrophilic loop and in TMS 6, and each membrane-embedded aromatic residue was mutated to cysteine. Out of the 41 constructed single cysteine mutants, only one mutant, L301C, was not expressed. Most single-cysteine mutants were capable of drug transport and only three mutants, F37C, M299C and N300C, were inactive, indicating that none of the aromatic residues in the transmembrane regions of LmrA is crucial for substrate binding or transport. Modification of the active mutants with *N*-ethylmaleimide blocked the transport activity in 5 mutants (S132C, L174C, S206C, S234C, and L292C). All cysteine residues in external and internal loops were accessible to fluorescein maleimide. The labeling experiments also showed that this thiol reagent can not cross the membrane under the conditions used, and confirmed the presence of 6 TMSs in each monomeric half of the transporter. Surprisingly, several single cysteines in the predicted TMSs could also be labeled by the bulky fluorescein maleimide molecule, suggesting unrestricted accessibility via an aqueous pathway. The periodicity of fluorescein maleimide accessibility of residues 291 to 308 in TMS 6 showed that this membrane spanning α-helix has one face of the helix exposed to an aqueous cavity along its full length. This finding, together with the solvent accessibility of 11 out of 15 membrane-embedded aromatic residues, indicates that the transmembrane domains of the LmrA transporter form, under non-energized conditions, an aqueous chamber within the membrane, which is open to the intracellular milieu.
INTRODUCTION

Resistance of cells to multiple structurally unrelated drugs and/or antibiotics (termed multidrug resistance) causes serious problems in the treatment of cancers and infectious diseases (1, 2). Multidrug resistance can be conferred by a number of transporters (MDRs\textsuperscript{1}) that pump drugs out of cells (for recent reviews, see 3, 4). Certain multidrug transporters, such as the \textit{Lactococcus lactis} protein LmrA, can transport an extreme diversity of structurally dissimilar drug molecules (reviewed in 5). LmrA belongs to a superfamily of transporters that contain an ATP binding cassette (ABC) (6). It shares significant sequence similarity with members of the P-glycoprotein subfamily of ABC transporters, most notably the human multidrug resistance P-glycoprotein (6). This human P-glycoprotein, which is also called MDR1, plays a crucial role in the resistance of cancer cells against cytotoxic agents used in chemotherapy (2). Bacterial LmrA can functionally complement P-glycoprotein in human lung fibroblast cells, and both proteins have a very similar drug and modulator specificity (7). This remarkable conservation of function between these two ABC-type multidrug transporters implies a common overall structure and transport mechanism (8).

All ABC transporters, described so far, show a four-domain organization and consist of two transmembrane domains (TMDs) and two ABC domains (also called nucleotide binding domains or NBDs). The TMDs are thought to form a pathway across the membrane through which solutes move. These domains consist of multiple membrane spanning segments (putative \(\alpha\)-helices) and contain the substrate binding sites. The other two domains are located at the cytoplasmic face of the membrane and couple ATP hydrolysis to substrate translocation. Conformational changes, induced by ATP binding and/or hydrolysis, are transmitted from the NBDs to the TMDs, which then most likely results in solute translocation. The four domains may be organized either in a multifunctional, single
polypeptide chain, as found for P-glycoprotein, or as separate proteins. LmrA appears to be a half-transporter consisting of an amino-terminal transmembrane domain with six predicted membrane spanning segments fused to an NBD domain (Fig. 1) (6). Several lines of evidence convincingly demonstrated that LmrA is functional as a homodimer, consistent with the general four-domain organization of ABC transporters (9).

Most ABC transporters recognize and transport highly hydrophilic substrates, including sugars and inorganic ions, most likely via an aqueous pathway. An example is the cystic fibrosis transmembrane regulator (CFTR), which unquestionably forms an aqueous pathway across the membrane through which chloride ions travel. However, multidrug transporters such as LmrA and P-glycoprotein translocate highly hydrophobic substrates and transport might occur via a hydrophobic, lipid-filled pathway. LmrA and P-glycoprotein have been shown to function as "hydrophobic vacuum cleaners" by extruding drug molecules from the inner leaflet of the lipid bilayer directly into the (external) aqueous phase (10, 11). These and other studies suggest that the translocation pathway has an opening to the extracellular milieu, and may be accessible from the membrane rather than from the internal aqueous phase.

In this study we used a cysteine scanning accessibility approach to obtain information about the structural properties of LmrA. Cysteines were introduced in the cysteine-less wild-type LmrA in each hydrophilic loop and in TMS 6, and each membrane-embedded aromatic residue was mutated to cysteine. The accessibility was studied of these cysteines to fluorescein maleimide, a well characterized sulfhydryl reagent that reacts with cysteines that are located in polar environments, but not with those in nonpolar environments. The accessibilities of cysteine replacements of all amino acid residues in TMS 6 were studied in detail as the corresponding TMSs in P-glycoprotein (TMSs 6 and 12) have been strongly implicated in substrate binding (e.g. 12, 13). The membrane-embedded aromatic residues
were chosen for study since aromatic rings can contribute to binding of aromatic cationic substrates through both aromatic-aromatic and π-cation interactions (14). In addition, aromatic residues lining an internal channel may provide a surface which is sterically compatible to the geometry of the drug and thus may promote the passage of a variety of ring-containing LmrA substrates (14). Taken as a whole, the results suggest the presence of a water-filled chamber, which in the non-energized form of LmrA is open to the intracellular environment, within the membrane-embedded domain of this transporter for hydrophobic molecules.

EXPERIMENTAL PROCEDURES

**Bacterial strains, plasmids, and growth conditions**

*Lactococcus lactis* NZ9000 (*lmrA*), which lacks the gene encoding multidrug transporter LmrA (obtained from O. Gajic and J. Kok, Department of Genetics, University of Groningen), was used in combination with the nisin-controlled expression (NICE) system (15, 16) for overexpression of LmrA and the single cysteine mutants. *L. lactis* NZ9700 (15) was used as a nisin-producing strain. The cells were grown at 30°C in M17 medium (Difco) supplemented with 0.5% glucose and 5 µg/ml chloramphenicol when appropriate. Expression of LmrA mutants from pNZ8048-derived plasmids (obtained from Dr. O. Kuipers, NIZO, The Netherlands) was induced by adding 40 pg/ml nisin at an OD$_{660}$ of about 0.8, and cells were harvested 1.5 h after induction.

**Site-directed mutagenesis**

Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described by Sambrook et
al. (17). The overlap extension polymerase chain reaction (18) was used to introduce mutations in the *lmrA* gene on plasmid pNHLmrA, which encodes LmrA with an amino-terminal His-tag consisting of six adjacent histidines (19). All polymerase chain reaction-amplified DNA fragments were sequenced to ensure that only the intended changes have been introduced. DNA sequencing was performed at the BioMedical Technology Centre (Groningen, The Netherlands).

**Preparation of membrane vesicles**

Membrane vesicles with an inside-out orientation were prepared by passage through a french pressure cell as described before (19). To prepare randomly oriented membrane vesicles, inside-out membrane vesicles were sonicated 3 times for 5 s on ice. The vesicles were frozen in liquid nitrogen and stored at -80°C at a protein concentration of 20 mg/ml in 50 mM Tris-HCl, pH 7.4, containing 10% glycerol. The protein concentration was determined with the DC protein assay from Bio-Rad. Expression levels of LmrA mutants were determined by analyzing membrane vesicles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions on gels containing 10% polyacrylamide. The gels were stained with Coomassie brilliant blue.

**Transport assays**

Inside-out membrane vesicles were diluted to a protein concentration of 0.5 mg/ml in 2 ml of 50 mM (K)Hepes, pH 7.4, supplemented with 2 mM MgSO₄, 8.5 mM NaCl, 0.1 mg/ml creatine kinase, and 5 mM phosphocreatine. After 1 min incubation at 30°C, 2 µM 2-[2-(4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl)-(4-piperazil)-benzimidazole (Hoechst 33342, Molecular Probes, Eugene, Oregon) was added and the partitioning of the drug into the membrane vesicles was followed fluorimetrically until a steady state was reached.
Subsequently, 2 mM Mg-ATP was added, and Hoechst 33342 fluorescence was followed as a function of time. Fluorescence was monitored with a Perkin-Elmer LS 50B fluorometer, using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 3 nm each.

To test the effect of thiol-reagents on transport, inside-out membrane vesicles (1 mg of total protein) were incubated for 10 min at 30ºC with 1 mM N-ethylmaleimide (NEM; Sigma) or 1 mM 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA; Antractace, Ohio). Immediately after labeling, Hoechst 33342 transport assays were performed with these membrane vesicles.

**Labeling of membrane vesicles with maleimides**

Unless stated otherwise, membrane vesicles (1 mg of total protein in 500 µl of 50 mM Tris-HCl, pH 7.4) were incubated in the presence of 100 µM fluorescein maleimide (Molecular Probes) for 15 min at 20ºC. The reaction was stopped by adding a 100-fold excess of dithiothreitol (DTT). When indicated, the reaction with fluorescein maleimide was preceded by incubation for 5 min at 20ºC with 1 mM NEM or 100 µM (2-(trimethylammonium)ethyl) methanethiosulfonate bromide (MTSET, Antractace).

Purification of His-tagged LmrA from membrane vesicles was done essentially as described (19) by using Ni²⁺-nitrilotriacetic acid agarose (Ni-NTA, Qiagen, Hilden, Germany). Purified proteins were run on SDS-polyacrylamide gels. Fluorescence of proteins labeled with fluorescein maleimide was visualized by UV excitation using a Lumi-imager (Roche).

**RESULTS**
Construction, expression, and activity of single cysteine mutants

Wild-type LmrA is devoid of native cysteine residues, and, therefore, is excellently suited for a cysteine-scanning mutagenesis approach. In this study, site-directed mutagenesis was used to construct a set of 41 single cysteine mutants. The codon for each residue in TMS 6 (residues 291-312) was mutated to that of cysteine, as well as the codon for each membrane-embedded aromatic residue (Fig. 1). In addition, at least one single cysteine was introduced in each hydrophilic loop of LmrA (Fig. 1). The single cysteine mutants were expressed in L. lactis NZ9000 (lmrA−), a strain in which the chromosomal lmrA gene was deleted. Out of the 41 cysteine mutants, 40 could be expressed and were present in the membrane, albeit at different levels (Fig. 2). In general, most mutations introduced at the positions of aromatic residues in the predicted membrane domain do not significantly effect the expression level (Fig. 2B), whereas most mutations introduced in the loop regions (Fig. 2C) or in TMS 6 (Fig. 2A) lower the expression level of LmrA. Mutant F37C (located in TMS 1) was observed to be highly susceptible to degradation suggesting that a major structural pertubation occurred upon substitution of this residue. Only one mutant, L301C, which is located in the middle of TMS 6, was not found in the membrane of L. lactis (Fig. 2A).

Interestingly, after SDS-PAGE of membranes containing mutant Y279C (Fig. 2B), a dominant protein band at about 130 kDa was observed. On the basis of its size, this band may represent an oxidized homodimer of LmrA. This hypothesis was tested by purifying His-tagged LmrA protein from membranes containing the Y279C mutant by Ni-NTA affinity chromatography. The putative cross-linked homodimer copurified with monomeric LmrA, indicating the presence of a His-tag on the oxidized dimer. In addition, this putative cross-linked dimer was detected by immunoblot analysis using anti-His-tag antibodies (data not shown). The cross-linked product disappeared upon incubation with DTT, concomitant with
an increase in the monomeric form of LmrA (not shown), confirming the disulfide nature of the cross-link. Taken together, these results demonstrate that spontaneous oxidation of the single cysteine at position 279 results in a cross-linked homodimeric LmrA molecule.

Previously, we have used Hoechst 33342 to monitor drug transport by wild-type LmrA in inside-out membrane-vesicles of *L. lactis* (19). The amphiphilic character and the high lipid-water partition coefficient of Hoechst 33342 result in partitioning of this drug into the lipid bilayer. Conveniently, Hoechst 33342 is strongly fluorescent when it resides in the lipid environment of the membrane, but is essentially non-fluorescent in the aqueous phase. These properties make it possible to follow fluorimetrically the presence of this compound in the membrane environment. ATP-dependent transport of Hoechst 33342 from the membrane of inside-out oriented membrane vesicles prepared from wild-type LmrA expressing NZ9000 (*lmrA*) cells resulted in a decrease in fluorescence (not shown). Such ATP-dependent transport of Hoechst 33342 was not observed in inside-out membrane vesicles prepared from NZ9000 (*lmrA*) cells not expressing LmrA.

At most of the positions of membrane-embedded aromatic residues, cysteine mutations were well tolerated by the transporter, resulting in similar or slightly reduced transport activities when compared to the wild-type protein (Table 1). The proteinase-sensitive mutant F37C was found to be inactive. Cysteine mutations in the loop regions of the transporter were all well tolerated (Table 1). Mutants S65C and T109C displayed wild-type transport activities, whereas mutants S132C, L174C, S206C, S234C, and S287C had slightly reduced transport activities. In contrast, several cysteine mutants in TMS 6 exhibited strongly reduced transport activities (Table 1). This might be explained, at least in part, by the low expression levels of these mutants (Fig. 2A). Two mutants in TMS 6, M299C and N300C, displayed no measurable transport activities.
We have shown previously that verapamil is an inhibitor of LmrA-mediated Hoechst 33342 transport (19). The ability of verapamil to inhibit transport by the single cysteine mutants was examined (Table 1). Verapamil (50 µM) inhibited Hoechst 33342 transport by wild-type LmrA and most mutants by more than 50%. In contrast, verapamil had only minor effects on Hoechst 33342 transport by mutants L298C, G303C, T307C, and A309C (Table 1), suggesting that these mutants have reduced affinity for the inhibitor.

**Labeling characteristics of intra- and extracellular cysteines.**

The fluorophore fluorescein maleimide reacts with cysteine residues in polar environments, but not with cysteines in nonpolar environments (20). Cysteines in putative transmembrane domains were found to be non-accessible to fluorescein maleimide, whereas cysteines in polar loop regions are readily labeled (e.g. 21, 22, 23). Fluorescein maleimide is therefore a suitable probe to determine whether the cysteine is situated in a polar loop region or within a lipidic membrane region.

It was first tested whether fluorescein maleimide and MTSET (see below) might be substrates of multidrug transporter LmrA. Treatment of wild-type LmrA with fluorescein maleimide (0.25 mM) or MTSET (1 mM) did not affect LmrA-mediated Hoechst 33342 transport, indicating that these hydrophilic thiol reagents are not recognized as substrates by the LmrA transporter. The accessibility of the single cysteine residues in the putative hydrophilic loops of LmrA to fluorescein maleimide was tested in randomly oriented membrane vesicles and in membrane vesicles with an inside-out orientation. His-tagged LmrA variants, solubilized from the membrane vesicles, were purified, and subjected to SDS-PAGE. The fluorescence of LmrA mutants labeled with fluorescein maleimide was detected by UV excitation. The wild-type, cysteine-less, LmrA was used as a negative control and was not modified by fluorescein maleimide in inside-out membrane vesicles (Fig. 3A). The single
cysteines at positions 109 and 132 (internal loop between TMS 2 and 3) and at positions 206 and 234 (internal loop between TMS 4 and 5) are located at the outside of inside-out membrane vesicles. These cysteines could be labeled by fluorescein maleimide in the membrane vesicles (Fig. 3A) and this labeling could be prevented by pre-incubation with the charged, membrane-impermeable thiol-reagent MTSET (data not shown). This behavior indicates that cysteines at positions 109, 132, 206, and 234 are accessible from an aqueous environment which is consistent with a cytoplasmic location of the cysteines. The single cysteines in mutants S65C (external loop between TMS 1 and 2), L174C (external loop between TMS 3 and 4), and S287C (external loop between TMS 5 and 6) are located at the inside of inside-out membrane vesicles. In these membrane vesicles, these cysteines could not be labeled by fluorescein maleimide (Fig. 3A). Even when fluorescein-maleimide labeling experiments with mutants S65C and S287C were repeated at higher temperatures and prolonged incubation times, no modification was observed (Fig. 3B). However, when these cysteine mutants were solubilized from inside-out membrane vesicles using the detergent n-dodecyl-β-D-maltoside, they were readily labeled by fluorescein maleimide (Fig. 3C). Also in randomly oriented membrane vesicles, prepared from inside-out membrane vesicles by sonication, mutants S65C, L174C, and S287C could be labeled by fluorescein maleimide (data not shown). When inside-out membrane vesicles containing mutants S65C, L174C, and S287C were first pre-incubated with MTSET and subsequently sonicated to generate randomly oriented membrane vesicles, all three cysteines could be labeled with fluorescein maleimide (data not shown). This behavior is in contrast to that of the cysteines at positions 109, 132, 206, and 234 and consistent with the extracellular location of the cysteines at positions 65, 174, and 287. These results furthermore demonstrate that fluorescein maleimide does not cross the membrane of inside-out vesicles under the conditions of the experiment.
Labeling characteristics of cysteines located in the predicted membrane domain

Using inside-out membrane vesicles, the accessibility of the single cysteine mutants in the region of TMS 6 to fluorescein maleimide was tested. Surprisingly, several of these cysteines were labeled by fluorescein maleimide (Fig. 4). Although these residues are predicted to reside within the membrane domain of LmrA, the accessibility of these residues for fluorescein maleimide indicates a location in the protein structure, which is well exposed to the aqueous environment. The cysteine residues in the mutants that are not labeled are either buried in the protein structure or exposed to the lipid environment. The periodicity of fluorescein maleimide accessibility (Fig. 4) strongly suggests that the region between Leu-291 and Val-308 spans the membrane as an \( \alpha \)-helix, which has one face of the helix accessible to an aqueous cavity. Indeed, when this region is modeled as an \( \alpha \)-helix, the solvent accessible residues cluster on one face of the helix (Fig. 5). The poorly expressed and transport inactive mutants also cluster on this face of the helix (Fig. 5).

From the labeling pattern of the cysteines located in the external and internal loops of LmrA (Fig. 3A), it appeared that fluorescein maleimide does not cross the membrane of inside-out vesicles. To confirm that the cysteine residues in helix 6 are accessible from the outside of inside-out membrane vesicles, the single cysteines were, prior to labeling with fluorescein maleimide, incubated with the charged and reportedly membrane-impermeable MTSET (24). In all cases, labeling by fluorescein maleimide was blocked by MTSET (Fig. 6), indicating that the cysteines in helix 6 are indeed accessible from the outside (corresponding to the cytoplasmic site of whole cells) of inside-out vesicles.

The fluorescein maleimide-reactive positions periodically align the full-length of TMS 6, suggesting that one side of this transmembrane helix faces a water-filled transmembrane chamber. Other parts of transmembrane regions of LmrA are also needed to compose such a water-filled chamber. To obtain information about potential other components of this chamber
the accessibility for fluorescein maleimide of single cysteines introduced at positions of the membrane-embedded aromatic residues was tested. At least one single cysteine residue present in each TMS could be labeled by fluorescein maleimide (Fig. 7), and this labeling was prevented by pre-incubation with MTSET (data not shown). These results suggest that several transmembrane segments of LmrA are involved in the formation of an aqueous chamber in the membrane that is accessible from the cytoplasm.

**Effect of thiol-reagents on transport activity**

The effect of cysteine modification on Hoechst 33342 transport was determined in inside-out membrane vesicles prepared from cells expressing the single cysteine mutants. Two small thiol-reagents were used: NEM and MTSEA, which are both membrane permeable. Treatment of wild-type LmrA with NEM or MTSEA did not affect Hoechst 33342 transport. Four single cysteine mutants (S132C, L174C, S206C, and S234C) in the putative loop regions of LmrA were completely inactivated upon incubation with NEM (data not shown). The other three cysteine mutants in the loop regions were not affected by NEM treatment. To our surprise, none of the single cysteine mutants at positions of membrane-embedded aromatic residues was affected by NEM or MTSEA (data not shown), and only one mutant (L292C) in TMS 6 was inactivated by NEM (data not shown).

The single cysteine mutants that were not affected by NEM either tolerated modification or could not be modified at all because they were not accessible. To distinguish between these two possibilities, the effect of NEM pre-treatment on labeling of the mutant proteins with fluorescein maleimide was determined. For all single cysteine mutants tested, pre-treatment with NEM prevents labeling by fluorescein maleimide, showing that these cysteine residues are accessible for NEM (Fig. 8, data not shown). The NEM-accessibility of mutants that could not be modified by fluorescein maleimide was not determined.
DISCUSSION

LmrA is an ABC multidrug transporter that mediates the extrusion of amphiphilic compounds from the inner leaflet of the membrane into the external water phase. The major physicochemical characteristics of the substrates of LmrA are: hydrophobic ring structure, presence of a basic amino group, and a high potency to partition within membranes. It is therefore most likely that the translocation path of LmrA is lined with non-polar residues (possibly aromatic side chains), in contrast to the translocation path of proteins that transport membrane-impermeable hydrophilic substrates. In this work we used a cysteine scanning mutagenesis approach in combination with thiol-modification techniques to obtain structural information about LmrA and to determine whether residues in the membrane-embedded domain of this transporter for hydrophobic molecules are accessible to the solvent.

To our surprise, only a very small number of residues appear to be essential to LmrA function. A set of 41 single cysteine mutants was constructed and nearly all mutants (37) showed a measurable transport activity. Only one protein of the inactive cysteine mutants (L301C) is not detectable at measurable levels, while two proteins (M299C and N300C) are poorly expressed, and one protein (F37C) is highly susceptible to proteinase activity. A role of these residues in catalytic activity is thus not evident. It is possible that they play a role in folding, insertion, or stability of the protein. It is noteworthy that cysteine replacements of residues in the middle of TMS 6 resulted in a drastic decrease in transport activity while replacements of membrane-embedded aromatic residues or residues in loop regions hardly affected the activity. Partially this might be explained by the reduced expression levels of these mutant proteins, but it also may reflect the role of TMS 6 in substrate binding. The finding that most residues of multidrug transporters can be replaced without serious impairment of function has previously been described and discussed for P-glycoprotein (25)
and the secondary multidrug transporter EmrE from *E. coli* (26). Importantly, it has also been observed that several single amino acid replacements in P-glycoprotein resulted into an altered substrate profile (25). Therefore, the role of the mutated residues in LmrA needs to be studied further by a more detailed characterization of the substrate specificity of the mutant proteins.

In our studies, mutagenesis of TMS 6 has generated proteins with modified specificity to at least one of the modulators of LmrA, which is most obvious for mutant A309C. This mutant displayed high Hoechst 33342 transport activity, but, in contrast to the wild-type protein, Hoechst 33342 transport was poorly inhibited by verapamil, suggesting that this mutant had lost the ability to bind verapamil with high affinity. Mutants L298C, G303C, and T307C also seem to be affected in their ability to bind verapamil. Loo and Clarke (27) defined the verapamil binding site in P-glycoprotein using cysteine scanning mutagenesis and thiol modification with a methanethiosulfonate analog of verapamil. Their results suggested a role of residues in TMSs 4, 6, 10, 11, and 12 in verapamil binding. Interestingly, one of these residues (G984 in TMS 12 of P-glycoprotein) is conserved as G303 in TMS 6 of LmrA. The decreased binding of verapamil to this mutant of LmrA suggests that at least some of the binding contacts for verapamil are conserved in human P-glycoprotein and bacterial LmrA. Further mutagenesis studies, which could be guided by the presumed anologies to the verapamil binding contacts in P-glycoprotein, are required to define all residues involved in binding of verapamil by LmrA.

In previous studies, fluorescein maleimide, which at neutral pH is a di-anion and membrane-impermeable, proved to be a useful fluorophore to determine the membrane topology of integral membrane proteins by modification of engineered cysteines (21, 22, 23). It was found that this thiol-reagent does not react with cysteines in the lipidic membrane environment, but readily reacts with cysteines in polar loop regions. Our results demonstrate
that engineered cysteines in the putative hydrophilic loops of LmrA are indeed accessible to fluorescein maleimide, as expected. The difference in labeling between inside-out and randomly oriented membrane vesicles was used to discriminate between an intracellular or extracellular location of the residues. Previous studies demonstrated that both the N- and C-terminus of LmrA face the cytosolic side of the membrane (19), while Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy studies (28) revealed a mainly α-helical structure of the TMD of LmrA. Our results support the presence of 6 TMSs in the membrane region of LmrA, and the periodicity of fluorescein maleimide accessibility of residues within TMS 6 shows that TMS 6 spans the membrane as an α-helix. TMS6 is a rather hydrophobic α-helix (H= 0.67) with the highest hydrophobicity at the site of tyrosine 297. To our surprise, the cysteine scanning accessibility studies demonstrated that several engineered cysteines in the membrane domain of LmrA are accessible to fluorescein maleimide. These reactive residues must therefore delineate water-filled cavities or, more likely, a water-filled chamber. Interestingly, the putative chamber in LmrA appears to be open to the intracellular environment.

In a previous publication, we proposed a two-cylinder engine model for the excretion mechanism of LmrA (9). According to this model two monomeric LmrA molecules form a functional dimer. Each monomer catalyses a transport cycle but the individual drug-binding sites of the monomers interact allosterically. A transport cycle of a monomer starts by binding of a lipophilic substrate to a high-affinity substrate-binding site exposed to the intracellular milieu. Subsequently, conformational changes induced by ATP-binding and/or hydrolysis result in an occluded state of this loaded substrate-binding site followed by an exposure of the then low-affinity binding site to the extracellular milieu where the substrate is released. According to the hydrophobic vacuum cleaner mechanism proposed for LmrA (10), the intracellular milieu is the inner leaflet of the membrane bilayer, whereas the extracellular.
milieu is the aqueous phase. Substrate translocation from the lipid phase to the aqueous phase could involve entering of the substrate in a lipid-filled chamber from the inner leaflet of the membrane bilayer, opening of the chamber to the extracellular milieu by an ATP-hydrolysis induced conformation change of the transporter and release of the substrate into the extracellular aqueous phase. However, the results presented above indicate the presence of a water-filled chamber within the LmrA transporter, which under non-energized conditions is exposed to the cytosol. This water-filled chamber is formed by one side of TMS 6 with contributions from other TMSs. The presence of this cytoplasmic exposed, water-filled chamber within the transporter implies that hydrophobic substrates are excreted from the membrane into the aqueous phase within the chamber, consistent with a vacuum cleaner mechanism, before being released into the extracellular aqueous phase. Significant conformational changes in LmrA upon hydrolysis of ATP have been detected by ATR-FTIR spectroscopy (28, 29). These conformational changes might lead to an opening of the water-filled chamber to the external surface as has been indicated by recent structural studies of a functional homolog of LmrA, P-glycoprotein (30). Projection structures of P-glycoprotein showed that the TMDs of this protein can form a chamber within the membrane that opens to the extracellular milieu. Apparently, P-glycoprotein was trapped in the crystals in a conformation distinct from the one analyzed here for LmrA. Thus, most likely the lipophilic substrate is released into an externally exposed, water-filled chamber, formed as a result of ATP hydrolysis-induced conformational changes, from which it exits the cell.

Taken the currently available data together, it appears that the TMDs of ABC multidrug (-like) transporters form a chamber within the membrane that alternates between at least two different conformations; one with the chamber exposed to the intracellular environment, and one in which the chamber has an opening to the extracellular environment. Our experimental results presented here and previous proton-deteurium exchange studies
combined with ATR-FTIR spectroscopy of LmrA (28) indicate that the chamber in these proteins is water-filled rather than lipid-filled.
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REFERENCES


FOOTNOTES

1The abbreviations used are: MDRs, multidrug resistance transporters; ABC, ATP binding cassette; TMD, transmembrane domain; NBD, nucleotide binding domain; TMS, transmembrane segment; CFTR, cystic fibrosis transmembrane regulator; NICE, nisin-controlled expression; His<sub>6</sub>-tag, tag of six adjacent histidine residues; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; DTT, dithiothreitol; MTSET, (2-(trimethylammonium)ethyl) methanethiosulfonate bromide; Ni-NTA, Ni<sup>2+</sup>-nitrilotriacetic acid agarose; ATR-FTIR, Attenuated Total Reflection-Fourier Transform InfraRed.
TABLE 1. Hoechst 33342 transport activities and verapamil inhibition of cysteine mutants of LmrA in inside-out membrane vesicles. For clarity of presentation, activities are presented as initial rates of transport relative to wild-type LmrA (100%). The initial rates were determined using data points from 10 to 50 s after addition of 2 mM Mg-ATP. +, verapamil (50 µM) inhibited Hoechst 33342 transport by more than 50%; -, verapamil inhibited Hoechst 33342 transport by less than 20%. i, not expressed.

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FIGURE LEGENDS

Fig. 1. Membrane topology of *L. lactis* multidrug transporter LmrA. Mutated positions are circled. Mutated positions in the loop regions are numbered. Putative TMSs are shown in boxes. NBD, nucleotide-binding domain.

Fig. 2. Expression of single cysteine mutants in *L. lactis* NZ9000 (*lmrA*). Comassie brilliant blue-stained SDS-PAGE gels of total membrane protein (30 µg of protein/lane) from inside-out membrane vesicles containing wild-type LmrA (WT) or mutant forms of LmrA with cysteine substitutions in the region of TMS 6 (A), at the positions of membrane-embedded aromatic residues (B), or in the loop regions (C). The arrows indicate the position of LmrA protein. The asterisk indicates the position of the cross-linked homodimeric LmrA molecule. The numbers above the lanes correspond to the amino acid numbering of LmrA.

Fig. 3. Modification of intra- and extracellular cysteines with fluorescein maleimide. (A), Membranes containing wild-type LmrA (WT) or single cysteine mutants were incubated with 100 µM fluorescein maleimide for 15 min at 20°C, followed by solubilization and purification of His6-tagged protein on Ni-NTA agarose. After purification, 5 µg of protein was subjected to SDS-PAGE. Labeled proteins were visualized with a Lumi-imager. (B), Membranes containing mutants S65C and S287C were incubated with 100 µM fluorescein maleimide for 15 min at 20°C (lanes 1 and 4), 15 min at 30°C (lanes 2 and 5), or 30 min at 30°C (lanes 3 and 6), followed by solubilization and purification of the proteins. Visualization of labeled protein was done as described above. Membranes containing mutant S132C were labeled with 100 µM fluorescein maleimide for 15 min at 20°C, and used as a positive control. (C) Incubation of mutants S65C and S287C with 100 µM fluorescein maleimide for 15 min at 20°C in
inside-out membrane vesicles (M) or as solubilized proteins (S). Visualization of labeled protein was done as described above.

Fig. 4. **Modification of cysteines located in the predicted TMS 6 with fluorescein maleimide.** Membranes containing wild-type LmrA (WT) or single cysteine mutants were incubated with 100 µM fluorescein maleimide for 15 min at 20°C, followed by solubilization and purification of His<sub>6</sub>-tagged protein on Ni-NTA agarose. After purification, 5 µg of protein was subjected to SDS-PAGE (lower panel). Labeled proteins were visualized with a Lumimager (upper panel). Mutants M299C and N300C, and mutant L301C were not analyzed as they are inactive or not expressed, respectively. Mutant V305C was not labeled by fluorescein maleimide (not shown).

Fig. 5. **Solvent accessible cysteine residues cluster on one face of transmembrane helix 6.** Amino acid positions within the predicted TMS 6 (arranged as α-helical wheel) of LmrA are shown and viewed from the cytoplasmic side of the membrane. Positions of residues that when mutated to cysteine are readily modified by fluorescein maleimide are circled. Asterisks indicate the positions of residues that when mutated to cysteine are inactive (positions 299 and 300) or not expressed (position 301).

Fig. 6. **Pre-incubation with MTSET prevents labeling by fluorescein maleimide.** Membranes containing single cysteine mutants were incubated with 100 µM fluorescein maleimide for 15 min at 20°C, followed by solubilization and purification of His<sub>6</sub>-tagged protein on Ni-NTA agarose. When indicated (+), the reaction with fluorescein maleimide was preceded by incubation for 5 min at 20°C with 100 µM MTSET. After purification, 5 µg of
protein was subjected to SDS-PAGE (lower panels). Labeled proteins were visualized with a Lumi-imager (upper panels).

Fig. 7. **Fluorescein maleimide labeling of LmrA variants with cysteine mutations at positions of membrane-embedded aromatic residues.** Membranes containing single cysteine mutants were incubated with 100 µM fluorescein maleimide for 15 min at 20°C, followed by solubilization and purification of His₆-tagged proteins on Ni-NTA agarose. After purification, 5 µg of protein was subjected to SDS-PAGE, and labeled proteins were visualized with a Lumi-imager. Membranes containing mutant S132C were used as a positive control.

Fig. 8. **Accessibility of engineered cysteines to NEM.** Membranes containing single cysteine mutants were incubated with 100 µM fluorescein maleimide for 15 min at 20°C, followed by solubilization and purification of His₆-tagged protein on Ni-NTA agarose. When indicated (+NEM), the reaction with fluorescein maleimide was preceded by incubation for 5 min at 20°C with 1 mM NEM. After purification, 5 µg of protein was subjected to SDS-PAGE (lower panels). Labeled proteins were visualized with a Lumi-imager (upper panels).
Poelarends et al, Figure 2
Poelarends et al, Figure 4
Poelarends et al, Figure 5
Poelarends et al, Figure 6

Image of a diagram showing protein expression levels for different mutations.
Poelarends et al, Figure 7
Poelarends et al, Figure 8

[Image of the figure showing protein bands for different mutants]
The transmembrane domains of the ABC multidrug transporter LmrA form a cytoplasmic exposed, aqueous chamber within the membrane

Gerrit J.. Poelarends and Wil N.. Konings

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