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Research Article

Proton motive force mediates a reorientation of the cytosolic domains of the multidrug transporter LmrP

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Abstract. LmrP from *Lactococcus lactis* is a 45-kDa membrane protein that confers resistance to a wide variety of lipophilic compounds by acting as a proton motive force-driven efflux pump. This study shows that both the proton motive force and ligand interaction alter the accessibility of cytosolic tryptophan residues to a hydrophilic quencher. The proton motive force mediates an increase of LmrP accessibility toward the external medium and results in higher drug binding. Residues Asp^{128} and Asp^{68}, from cytosolic loops, are involved in the proton motive force-mediated accessibility change. Ligand binding does not modify the protein accessibility, but the proton motive force-mediated restructuring is prerequisite for a subsequent accessibility change mediated by ligand binding. Asp^{142} cooperates with other membrane-embedded carboxylic residues to promote a conformational change that increases LmrP accessibility toward: the hydrophilic quencher. This drug binding-mediated reorganization may be related to the transition between the high- and low-affinity drug-binding sites and is crucial for drug release in the extracellular medium.

Key words. Accessibility change; LmrP; multidrug resistance; proton motive force.

Resistance to a wide range of cytotoxic compounds is a common phenomenon observed in many organisms ranging from bacteria to humans [1, 2]. One important mechanism that organisms use to evade the lethal effect of cytotoxic drugs is the active efflux of the toxic compounds out of the cell through the action of transporters. In contrast to specific drug efflux systems, multidrug resistance (MDR) transporters can extrude a wide variety of structurally unrelated compounds. The structural basis of their broad chemical specificity is fairly well understood [3–5] but the mechanism of the energy coupling to the drug transport still awaits elucidation.

Most known bacterial MDR transporters are secondary transporters that use the proton motive force (pmf) to drive the excretion of drugs. Secondary MDR transporters belong to one of three distinct families of transport proteins which are distinguished on the basis of their structure: the major facilitator superfamily (MFS) [6], the resistance-nodulation-division family (RND) [7], and the small multidrug resistance family (SMR) [8]. In Gram-positive *Lactococcus lactis*, several drug extrusion activities have been detected, among which is the pmf-driven extrusion of cationic drugs mediated by the MDR transporter LmrP [9–11]. LmrP is a 408-amino-acid integral membrane protein that belongs to the MFS. Extrusion of drugs by LmrP is driven by both the membrane potential and the transmembrane proton gradient (ΔpH), indicating that LmrP

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mediates an electrogenic proton/drug antiport reaction. The number of compounds recognized and transported by LmrP is remarkably broad and includes many structurally diverse cytotoxic drugs such as lincosamides, macrolides, streptogramins, and tetracyclines [12]. A common property of most known substrates of LmrP is their ability to intercalate into the lipid bilayer because of their lipophilicity. This led to the hypothesis that LmrP recognizes its substrates within the membrane and not from the cytoplasm. Such a mechanism has been confirmed by transport experiments with the fluorescent membrane probe 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene [13]. The presence of multiple drug-binding sites in LmrP has been reported [14] and can explain the large range of potential drug-protein interactions.

To mediate drug binding in the membrane and subsequent release in the external medium, LmrP must pass through at least two configurations: a high-affinity membrane-facing drug-binding site and a low-affinity, outside-facing site for drug release. High-resolution structures of the *Escherichia coli* MDR transporter AcrB in its ligand-free and ligand-bound states [5, 15] revealed that substrate binding only mediates small conformational changes. This suggests that the proton flux is needed to mediate the large conformational changes required for drug extrusion by ion-coupled transporters [5]. Such an influence of the pmf on transporter structures can not be detected in crystal structures. Our study brings the first experimental evidence that a ΔpH imposed across the lipidic bilayer indeed modifies the accessibility of a MDR transporter to a hydrophilic quencher. This restructuring results in an increase in the drug affinity of LmrP and is necessary for subsequent reorientation mediated by drug binding. This drug binding-mediated reorganization may be related to the transition between the high- and low-affinity drug-binding sites described above and hence be crucial for drug release in the extracellular medium.

Comprehension of the LmrP transport requires a better knowledge of the coupling of H^+ to substrate translocation. The importance of charged residues in transmembrane domains in such transport activity has been described in several MDR ion-coupled transporters [4, 16, 17]. In EmrE from *E. coli*, a single membrane-embedded charged carboxyl residue is part of a common binding site for substrates and protons [18]. Therefore, EmrE shows a simple mode of coupling and has been proposed as a model system. However, other MDR ion-coupled systems may require a more complex mode of coupling proton to substrate translocation. LmrP mutagenesis experiments have shown that several carboxylic residues located in membrane helices and also extramembrane loops are crucial to preserve LmrP transport activity [19]. Whether these residues are essential to preserve LmrP structure, to modulate its substrate affinity, or to induce conformational changes involved in the proton-coupled drug transport is unknown. In this study, we demonstrate that extramembrane carboxylic residues Asp^{68} and Asp^{128} are essential to preserve the drastic and essential pmf-mediated reorganization, and that the extramembrane Asp^{68} modulates ligand binding, in addition to transmembrane residues such as Asp^{142}. After ligand binding, Asp^{142} cooperates with other membrane-embedded residues to promote the accessibility towards the hydrophilic quencher.

Materials and methods

Materials

Ni^{2+}-nitrilotriacetic acid (Ni-NTA)-agarose was from Qiagen; *E. coli* lipids and egg yolk phosphatidylcholine were from Avanti Polar Lipids; dodecyl-*β*-D-maltoside (DDM) was from Anatrace; pyranine, dicyclohexylcarbodiimide (DCCD), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), Triton X-100, verapamil, tetracycline, valinomycin, and ethidium bromide were from Sigma-Aldrich; acrylamide was from Merck; vinblastine was from Fluka; [^{3}H]tetracycline was from Perkin-Elmer Life Sciences, and Hoechst 33342 was from Molecular Probes.

Bacterial strains and growth conditions

L. lactis NZ9000 (Δ*lmrA*) [20] which lacks the gene encoding ATP-binding cassette-type MDR transporter LmrA (a kind gift from O. Gajic and J. Kok, Department of Genetics, University of Groningen) was used in combination with the NICE system [21, 22] for overexpression of wild type (WT) and D68C, D128C and D142A mutants of LmrP [19, 23, 24]. *L. lactis* cells were grown at 30 °C in M17 medium (Difco) supplemented with 0.5 % (w/v) glucose and 5 μg/ml chloramphenicol. Expression of LmrP variants was induced by adding approximately 10 ng nisin A per milliliter at an A_{660} of about 0.6, and cells were harvested 60 min after induction.

Purification and reconstitution of LmrP

His-tagged WT or mutated LmrP were purified by affinity chromatography using Ni-NTA-agarose as described elsewhere [19]. Briefly, solubilized membrane proteins were mixed with Ni-NTA resin (~25 μl resin/mg of protein), which was equilibrated with 50 mM potassium phosphate (pH 8.0) supplemented with 100 mM NaCl, 10% (v/v) glycerol, 0.2 mg/ml *E. coli* lipids, 0.05% detergent (buffer A), and 10 mM imidazole, and gently shaken for 60 min at 4 °C. The resin was transferred to a Bio-spin column (Bio-Rad) and subsequently washed with 20 column volumes of buffer A and 10 mM imidazole and 10 column volumes of buffer A and 20 mM imidazole. The protein was eluted with buffer A (pH 7.0) containing 250 mM imidazole, and fractions of 500 μl were collected.

A dried film of 1.5 mg *E. coli* lipids and egg yolk phosphatidylcholine in a 3 : 1 ratio (w/w) was obtained by evaporation of chloroform under a flow of nitrogen followed by overnight drying under vacuum. Liposomes were prepared by sonication of the lipid film for 5–8 min on a 250-W Vibra Cell Sonifier in 2,6 ml of 10 mM Tris pH 7.4, 0.5 mM EDTA, 1 mM DTT, 75 mM NaCl. DDM was added to the sonicated lipid suspension to obtain a 1 : 2 (w/w) lipid/detergent ratio. The purified LmrP protein was mixed with the detergent-saturated liposomes at a protein to lipid ratio of 1:10 (w/w) and incubated for 30 min at room temperature under gentle agitation. The detergent was then removed by absorption on SM2 Bio-Beads (three incubations of 2 h under continuous stirring in the presence 80 mg Bio-Beads). To demonstrate the association of proteins with lipids, the supernatant collected from the Bio-Beads mixed with an equal volume of 80% sucrose was overlaid with a 30 to 5% sucrose linear gradient and centrifuged overnight at 120,000 g and 4 °C in a Beckman L7 ultracentrifuge with a SW60 rotor. The gradient was fractionated and phospholipid and protein distributions along the gradient were determined by enzymatic colorimetric assays of the phosphatidylcholine and tryptophan fluorescence, respectively. To eliminate the sucrose, the fractions containing the proteoliposomes were washed twice by centrifugation in 3 mM Hepes, pH 7.4 for 2 h at 150,000 g and then resuspended in ~20 µl of the same buffer.

Imposition of a ΔpH across LmrP-containing liposomes

To impose an artificial proton gradient (ΔpH), proteoliposomes were collected by centrifugation (18,000 g for 30 min at 4 °C), resuspended in 20 mM potassium phosphate (pH 7.0) supplemented with 100 mM potassium acetate and 2 mM $MgSO_4$ (buffer I, final pH 7.8), followed by freezelthawing steps in liquid nitrogen. The frozen proteoliposomes were thawed at room temperature, collected by centrifugation (18,000 g for 30 min at 4 °C), and resuspended to a final concentration of 0.5 mg protein/ml in buffer I. A 10 µl sample was diluted 100-fold into 20 mM potassium phosphate (pH 7.0) containing 50 mM K_2SO_4 and 2 mM $MgSO_4$ (buffer II, final pH 6.9) plus 0.5 µM valinomycin to collapse the electrical potential.

The ΔpH across the proteoliposomal membranes was monitored by measuring the pH-sensitive fluorescence of pyranine [25]. Pyranine was added in buffer I at a final concentration of 0.5 mM and encapsulated into liposomes during the freeze-thawing steps described above to generate the *Δ*pH. External pyranine was removed by extensive washing of the proteoliposomes. The internal pH was followed by measuring the pyranine fluorescence (excitation at 461 ± 4 nm, emission 511 ± 4 nm) after dilution of 10 µl proteoliposomes into buffer II. Fluorescence after complete dissipation of *Δ*pH was determined by addition of 0.5% Triton X-100 into the liposome suspension. A correlation between pyranine fluorescence and pH was made by measuring the fluorescence of pyranine in buffers at pH values ranging between 6.9 and 7.8.

Hoechst 33342 transport in proteoliposomes

Hoechst 33342 transport in proteoliposomes containing about 6 µg WT and mutated LmrP was driven by an artificially imposed ΔpH (inside alkaline) as explained previously. Hoechst 33342 in water was added at a final concentration of 1 µM to the liposome suspension. In carbodiimide labeling experiments, 100 µM of DCCD or EDAC was added to the proteoliposome suspension. The fluorescence was monitored on a SLM Aminco 8000 fluorimeter using excitation and emission wavelengths of 355 and 457 nm, respectively.

Structural analysis of reconstituted LmrP by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra were recorded, at room temperature, on a Bruker IFS55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector at a nominal resolution of 2 cm^{-1}, and encoded every 1 cm^{-1}. The spectrophotometer was continuously purged with air dried on an FTIR purge gas generator 75–62 Balston at a flow rate of 5.8 l/min. The internal reflection element (ATR) was a germanium plate (50×20×2 mm) with an aperture angle of 45°, yielding 25 internal reflections [26].

The sample contained 20 µg reconstituted WT or mutated LmrP. Thin films of oriented multilayers were then obtained by slowly evaporating the sample on one side of the ATR plate, under a stream of nitrogen [26, 27]. The ATR plate was then sealed in a universal sample holder.

The sample on the ATR plate was rehydrated by flushing D_2O-satured N_2 for 2 h, at room temperature. A total of 512 scans were averaged for each measurement. Determination of the secondary structure was based on the vibrational bands of the protein and particularly the amide I band (1600–1700 cm^{-1}), which is sensitive to the secondary structure [28]. This amide I band, located in a region of the spectrum which is often free of other bands, is composed of 80% pure C=O vibration. The analysis was performed on the amide I region of deuterated samples because the H/D exchange allows differentiation of the α-helical secondary structure from the random secondary structure whose absorption band shifts from about 1655 cm^{-1} to about 1642 cm^{-1} [29]. Fourier self-deconvolution was applied to increase the resolution of the spectra in the amide I region. The self-deconvolution was carried out using a Lorentzian line shape for the deconvolution and a Gaussian line shape for the apodization [28]. To quantify the area of the different components of amide I revealed

by self-deconvolution, a least-squares iterative curve fitting was performed to fit Lorentzian line shapes to the spectrum between 1700 and 1600 cm^{-1} [30]. To avoid the introduction of artifacts due to the self-deconvolution procedure, the fitting was performed on the non-deconvoluted spectrum. The proportion of a particular structure is the sum of the area of all the fitted Lorentzian bands with their maximum in the frequency region where that structure occurs divided by the total area of the amide I. The frequency limits for each structure were first assigned according to theoretical [31] or experimental [32] data: 1662–1645 cm^{-1}, α-helix; 1689–1682 and 1637–1613 cm^{-1}, β sheet; 1644.5–1637 cm^{-1}, random; 1682–1662.5 cm^{-1}, β turns. These limits have been slightly adjusted to obtain a good agreement between the proportion of each structure determined by ATR-FTIR and X-ray crystallography.

Tryptophan fluorescence quenching by acrylamide

Acrylamide quenching experiments were carried out on an SLM Aminco 8000 fluorimeter at an excitation wavelength of 290 nm to reduce the absorbance of acrylamide. Acrylamide was added from a 3 M solution to the proteoliposome suspension containing 6 µg of reconstituted wild-type or mutated LmrP in the presence or absence of the various ligands (tetracycline, Hoechst 33342, ethidium bromide, vinblastine and verapamil) and/or the carbodiimides (EDAC and DCCD) at a final concentration of 10 µM and 100 µM, respectively. Fluorescence intensities were measured at 333 nm after each 5-µl addition of quencher. Acrylamide quenching data were analyzed according to the Stern-Volmer equation for collisional quenching [33]:

$$F_0/F = 1 + K[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher, and K is the Stern-Volmer quenching constant. Data were subjected to a linear fit up to 100 mM acrylamide. Above this concentration, the static quenching by acrylamide causes a deviation from linearity in Stern-Volmer plots.

Photoaffinity labeling by [^{3}H]tetracycline

Proteoliposomes containing ~10 µg WT or mutated LmrP (in buffer I) were collected by centrifugation and resuspended in 90 µl buffer II to impose an artificial ΔpH. For experiments carried out without ΔpH, the proteoliposome were prepared in buffer at a pH ranging from 6 to 8.5 by freeze/thaw steps and resuspended in the same buffer. [^{3}H]tetracycline at a final concentration of 10 µM was added to the proteoliposome suspension, in the presence or absence of the carbodiimides (EDAC and DCCD) at a final concentration of 100 µM. After irradiation with 365 nm UV light for 30 min, 900 µl buffer containing 1 mM of unlabeled tetracycline was added to stop the labeling reaction. Proteoliposomes were collected and washed in the appropriate buffer by ultracentrifugation at 18,000 g for 30 min at 4 °C. Proteoliposomes were resuspended in the appropriate buffer and analyzed by SDS-polyacrylamide gel electrophoresis. After Coomassie blue staining, the LmrP-containing band was cut and digested in 900 µl 30% H_2O_2 at 50 °C in capped vials overnight. Ten milliliters of scintillation fluid was added and the total radioactivity was measured by a Packard scintillation counter; 100% radioactivity was assigned to the maximal detected radioactivity whereas 0% was assigned to the radioactivity detected in a portion of polyacrylamide gel on the lane of protein migration.

Results

Role of membrane-embedded and extramembrane carboxylic residues in pmf-driven transport activity of reconstituted LmrP

WT and mutated LmrP were reconstituted by mixing purified proteins with DDM-destabilized *E. coli*/phosphatidylcholine vesicles and detergent removal by adsorption on polystyrene beads, essentially as described previously [19]. Proteoliposomes were layered at the bottom of a linear sucrose gradient and after centrifugation, the gradient was fractionated from the bottom of the tube. Comigration of the proteins and the lipids as a single peak demonstrated that proteoliposomes were formed (data not shown). The proteoliposome population was homogeneous and no aggregates were detectable at the bottom of the gradient.

To detect pmf-dependent transport activity of LmrP, a ΔpH of 0.9 pH units (interior alkaline) was imposed across the proteoliposomes as described in Materials and methods. The pH-sensitive probe pyranine was used to follow the time-dependent dissipation of this gradient [25]. The ΔpH dissipates slowly and reaches half its initial value after 30 min (fig. 1). All subsequent experiments carried out after imposition of a ΔpH were monitored within a period of 30 min.

The positively charged Hoechst 33342 is a substrate for LmrP [11] that is particularly useful in transport measurements because it is highly fluorescent when intercalated into the lipid membranes, but virtually non-fluorescent in aqueous solution. LmrP-mediated transport was assayed by measuring the variation in Hoechst 33342 fluorescence as a function of time. Hoechst 33342 transport does not occur in proteoliposomes in the absence of a ΔpH. However, when a ΔpH is imposed, Hoechst 33342 is actively extruded out of the membrane (fig. 2A). No transport activity was detected in liposomes that did not contain LmrP, whether a ΔpH was imposed or not (fig. 2A).

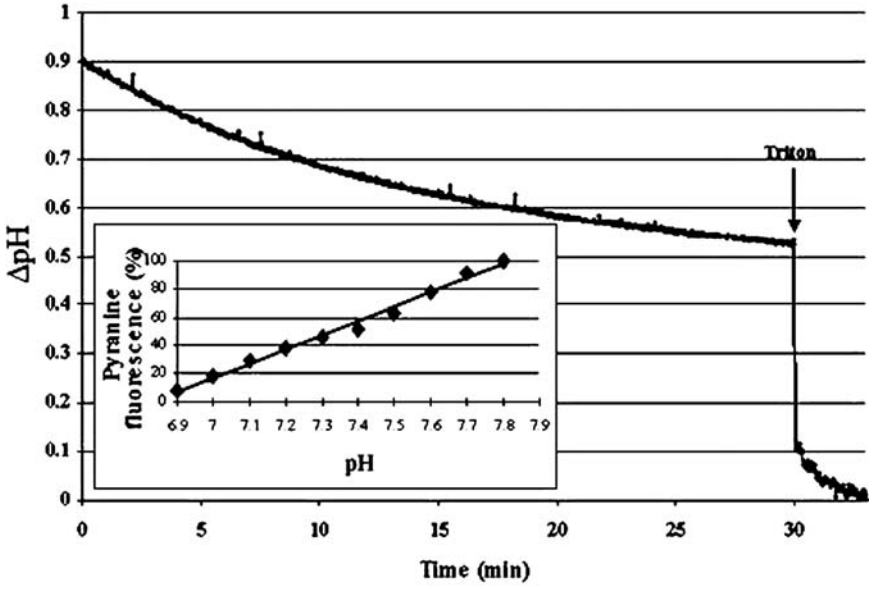

Figure 1. Measurement of the imposed ΔpH across LmrP-containing liposomes loaded with pyranine. Pyranine was entrapped in liposomes and a ΔpH (interior alkaline, ΔpH = 0.9) imposed as described in Materials and methods Gradient dissipation as a function of time was evaluated from the pH-dependent pyranine fluorescence shown in the inset (λ_{exc} = 461 nm, λ_{em} = 511 nm). After 30 min, Triton (0.5%) was added to solubilize liposomes, and pyranine fluorescence was measured after complete dissipation of the ΔpH.

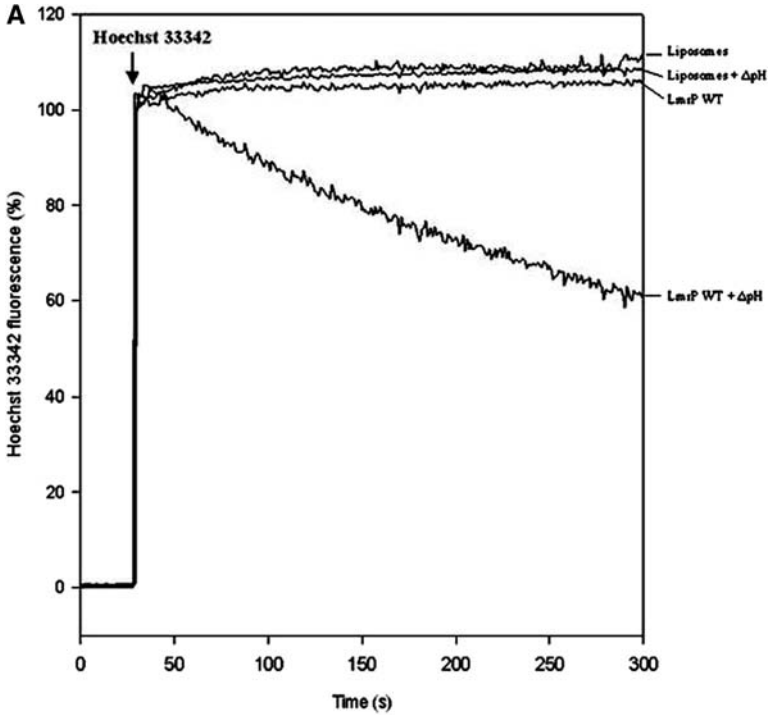

Figure 2. Hoechst 33342 transport in LmrP reconstituted into liposomes. Liposomes with or without inserted WT and mutated LmrP were diluted 100-fold in the buffer used for reconstitution. ΔpH across liposomes was imposed as described in Materials and methods. After 30 s of data acquisition (λ_{exc} = 355 nm and λ_{em} = 457 nm), 5 µl Hoechst 33342 200 µM was added. Fluorescence intensity after addition of Hoechst 33342 was normalized to 100%. Fluorescence was monitored for ~250 s. Carbodiimide EDAC (*C*) or DCCD (*B*) were added to the proteoliposome suspension at a 100 µM final concentration. When Hoechst 33342 transport was measured on (*A*) liposomes without inserted LmrP, with WT- or (*C*) mutant D68C-, D128C- or (*B*) D142A-inserted LmrP, traces are annotated 'Liposomes', 'LmrP WT', 'LmrP D68C', 'LmrP D128C' or 'LmrP D142A' respectively. 'ΔpH' is annotated when a ΔpH was imposed across the liposomes. Addition of EDAC or DCCD is annotated as '+EDAC' or '+DCCD', respectively.

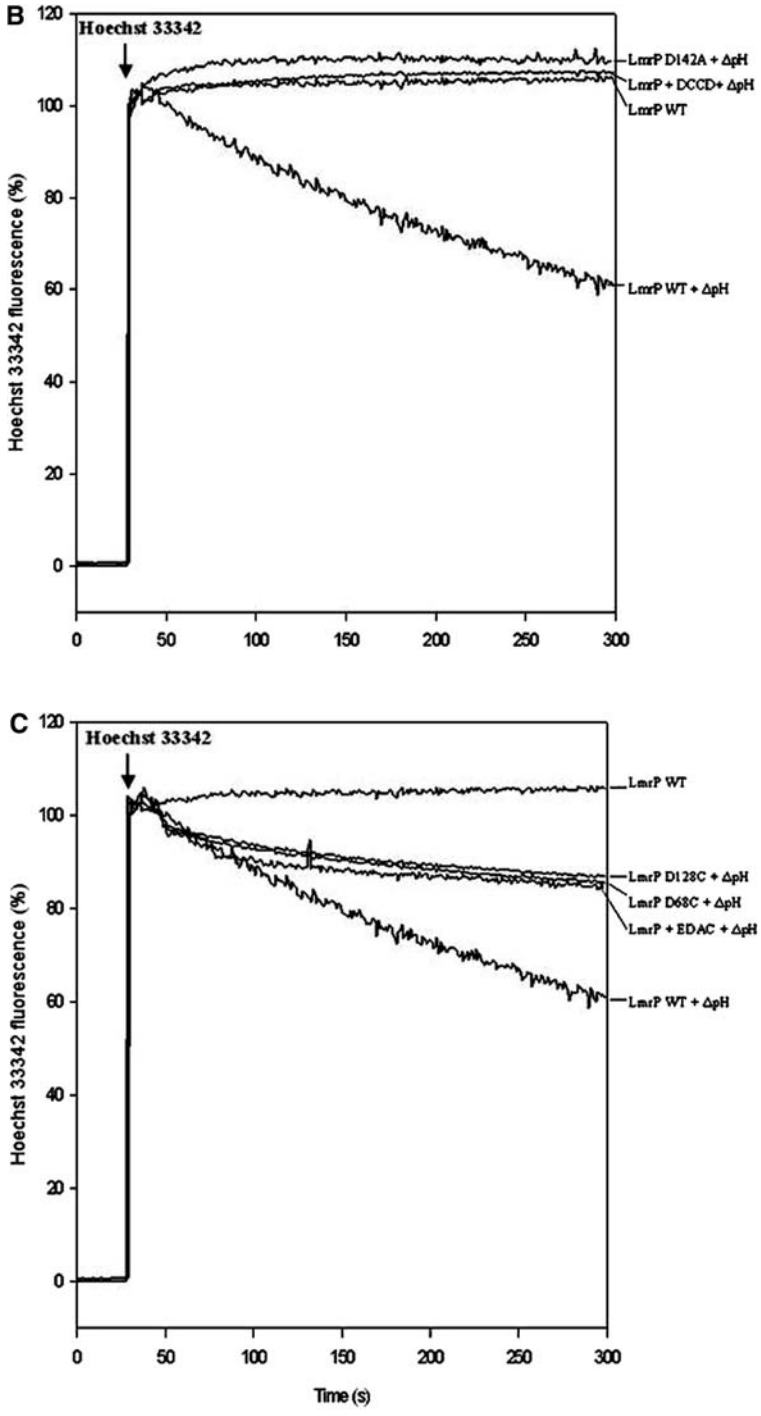

Figure 2 (continued)

The role of carboxylic residues in LmrP-mediated transport was characterized by carbodiimide chemical modification and mutation of membrane-embedded (D142A) and extramembrane carboxylic residues (D68C, D128C). Addition of DCCD, which reacts with carboxylic groups located in a hydrophobic environment [34, 35], inhibited LmrP-mediated transport of Hoechst 33342 (fig. 2B). This observation demonstrates the crucial role of some carboxylic residues located in a hydrophobic, probably membrane-embedded environment. No transport activity was indeed detected with mutant D142A, suggesting that at least this membrane carboxylic residue is necessary for the proper function of LmrP. After addition of EDAC, which reacts with carboxylic groups in hydrophilic environments [34, 35], the transport process was significantly decreased but still detectable (fig. 2C). This suggests that some carboxylic residues located in a hydrophilic environment, probably

in extramembrane loops, are not essential but affect the efficiency of the LmrP transport process. With mutants D128C and D68C, inhibition of Hoechst transport activity was indeed identical to that observed for the WT protein in the presence of EDAC (fig. 2C).

Mutations introduced in a protein may affect its folding and can lead to a partial or complete loss of activity even if the mutated residue has no functional role in the transport process. To verify whether mutations D142A, D68C and D128C affect LmrP in such a way, the global secondary-structure content of WT and mutated proteins was compared. We used ATR-FTIR spectroscopy to obtain information about the secondary structure of LmrP by analysis of the amide I band [due to the υ(C=O) vibration of the peptide bonds] of a deuterated sample as previously described [29]. No change in the amide I shape, and hence in the secondary structure of the transporter, was observed after introducing the mutations D142A, D68C or D128C, indicating that the global structure of LmrP remained unchanged. The effect of these mutations on the transport activity of LmrP therefore suggests a functional role for these residues. Moreover, the structural analysis agreed remarkably well with the predicted model [11] as it yielded an α-helical estimate of ~60%, corresponding to 12 transmembrane α helices. The remaining amino acid residues appear mostly in random structures (30%), with a minor amount in β sheets (10%), consistent with the expected structure of the extramembrane connecting loops.

Tryptophan accessibility is modified by imposition of a pmf and by ligand binding

LmrP contains seven tryptophan residues, four of which are predicted to be in extramembrane domains [11]. Trp exposure to the external solvent was determined by monitoring fluorescence intensity in the presence of increasing concentrations (0–100 mM) of the aqueous quencher acrylamide [33]. Substrates (tetracycline, ethidium bromide, Hoechst 33342) and inhibitors (verapamil and vinblastine) [12] were added to proteoliposomes containing reconstituted LmrP at a final concentration of 10 μM in the presence or absence of an artificial ΔpH. Data were analyzed according to the Stern-Volmer equation, as described in Materials and methods.

A first conformation (fig. 3.A; $K_{sv} = 3.07 \pm 0.03$) was observed when both sides of the liposomes were set at a similar pH (6.9 or 7.8). When a ΔpH (ΔpH = 0.9, interior alkaline) was imposed, the accessibility of the Trp residues toward the quencher was increased ($K_{sv} = 3.73 \pm 0.14$). This observation demonstrates that the pmf modifies the accessibility of the tryptophan residues located in the cytosolic loops. This change mediated by the pmf still occurred in the presence of DCCD, which reacts with carboxylic groups located in hydrophobic environments (fig. 3A). However, this effect was completely abolished when EDAC, a carboxylic group reagent that acts in hydrophilic environments, was used. These observations demonstrate that some carboxyl residues located in a hydrophilic environment, probably in extramembrane loops, are required in the pmf-mediated reorganization. Indeed, in mutants D68C and D128C, the change mediated by the pmf was not detectable (fig. 3B).

When no ΔpH was imposed (pH 6.9 or 7.8 at both sides of the membrane), LmrP accessibility was not modified by the presence of ligands (fig. 3C). This corroborates observations made on three-dimensional crystals of AcrB [5]. However, in the presence of a ΔpH, ligand binding resulted in an increase in Trp residue accessibility toward the quencher ($K_{sv} = 4.55 \pm 0.16$; fig. 3C). In D142A LmrP (fig. 3E), the accessibility of Trp toward the external medium was still modified by ligand binding but to a lesser extent than in the WT LmrP ($K_{sv} = 4.09 \pm 0.08$; fig. 3E). In contrast, after reaction of DCCD with LmrP, the ligand binding-mediated reorganization was completely inhibited. Indeed, the accessibility change was similar to that mediated by the ΔpH only (fig. 3C). Taken together, these observations suggest that DCCD reacts with other residues than Asp^{142} and that ligand binding-mediated restructuring most likely requires several membrane-embedded carboxylic residues. Another possibility is that modification of Asp^{142} by DCCD modifies the normal folding of LmrP. This hypothesis seems, however, unlikely. Indeed, structural analysis by ATR-FTIR of WT LmrP shows no significant modification of the secondary structure after reaction with DCCD (data not shown). When the accessibility change mediated by the pmf was prevented either by adding EDAC or by mutation of residues Asp^{68} or Asp^{128}, the ligand-mediated restructuring was also abolished (fig. 3C, D). This demonstrates that the pmf-mediated reorganization is necessary for the subsequent ligand binding-driven conformational changes.

Ligand binding to reconstituted LmrP determined by [^{3}H]tetracycline photolabeling

To identify some of the determinants responsible for drug affinity modifications during the LmrP-mediated transport process, the binding properties of LmrP to tetracycline, a substrate of LmrP [12], were characterized by photolabeling. Tetracycline is photoactive and has been used previously for determining the tetracycline binding to the metal-tetracycline/H^+ antiporter TetA [36]. Briefly, reconstituted LmrP was incubated with [^{3}H]tetracycline and then irradiated with UV light for 30 min. Liposomes were analyzed by SDS-PAGE and Coomassie blue staining. The band corresponding to LmrP was cut out of the gel and its radioactivity was measured. Irradiation of proteoliposomes containing LmrP with UV light for 30 min in the presence of [^{3}H]tetracycline resulted in LmrP-associated radioactivity (fig. 4A). In contrast, when lipo-

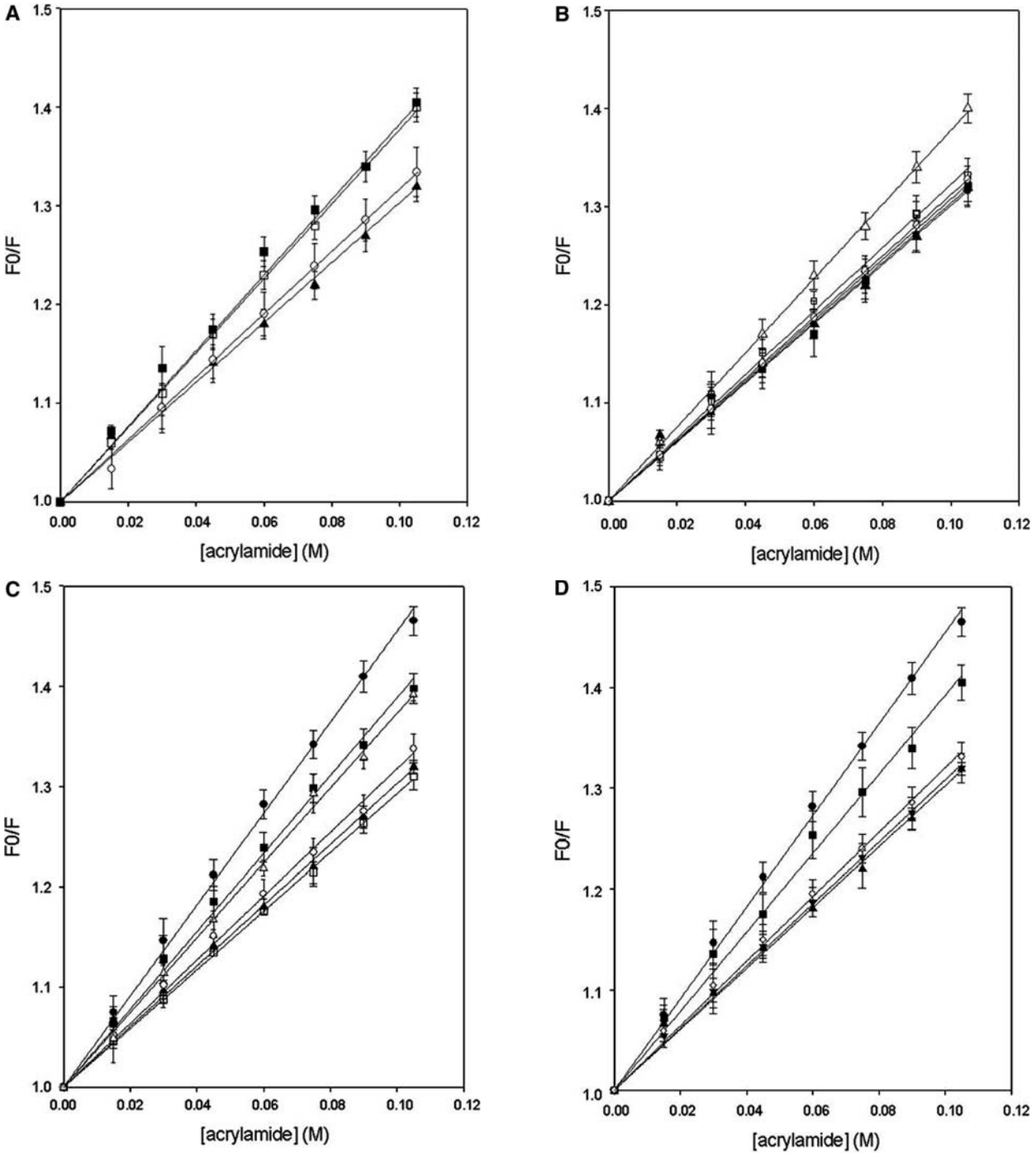

Figure 3. Stern-Volmer plots of tryptophan quenching by acrylamide in reconstituted LmrP. F is the measured Trp fluorescence intensity and F0 is the fluorescence in the absence of acrylamide. Experiments were completed within 5 min, and the dissipation of an imposed ΔpH is very small. The curves are the means of at least three experiments. (*A*) WT LmrP without imposition of a ΔpH (▲); WT LmrP after imposition of a ΔpH (■); WT LmrP after imposition of a ΔpH in the presence of 100 μM EDAC (○); WT LmrP after imposition of a ΔpH in the presence of 100 μM DCCD (□). (*B*) WT LmrP without imposition of a ΔpH (▲); WT LmrP after imposition of a ΔpH (△); D68C LmrP without imposition of a ΔpH (●); D68C LmrP after imposition of a ΔpH (○); D128C LmrP without imposition of a ΔpH (■); D128C LmrP after imposition of a ΔpH (□). (*C*) WT LmrP without imposition of a ΔpH (▲); WT LmrP after imposition of a ΔpH (■); WT LmrP without imposition of a ΔpH in the presence of 10 μM substrates (□); WT LmrP after imposition of a ΔpH in the presence of 10 μM substrates (●); WT LmrP after imposition of a ΔpH in the presence of 10 μM substrates and 100 μM DCCD (△); WT LmrP after imposition of a ΔpH in the presence of 10 μM substrates and 100 μM EDAC (○). (*D*) WT LmrP without imposition of a ΔpH (▲); WT LmrP after imposition of a ΔpH (■); WT LmrP after imposition of a ΔpH in the presence of 10 μM substrates (●); D68C LmrP after imposition of a ΔpH in the presence of 10 μM substrates (▼); D128C LmrP after imposition of a ΔpH in the presence of 10 μM substrates (○).

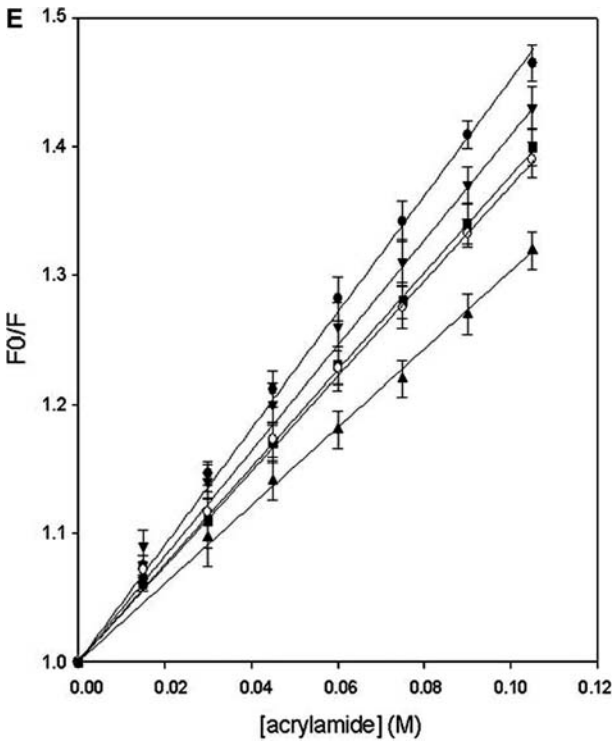

Figure 3 (continued) (*E*) WT LmrP without imposition of a ΔpH (▲); WT LmrP after imposition of a ΔpH (■); WT LmrP after imposition of a ΔpH in the presence of 10 μM substrates (●); D142A LmrP after imposition of a ΔpH (○); D142A LmrP after imposition of a ΔpH in the presence of 10 μM substrates (▼).

somes that did not contain LmrP were incubated in the presence of [^{3}H]tetracycline and irradiated, no significant radioactivity was detected. These observations demonstrate that tetracycline binds specifically to reconstituted LmrP.

To determine the pH dependence of tetracycline binding to LmrP, [^{3}H] tetracycline labeling was carried out in a pH range from 6 to 8.5. Tetracycline labeling increased from pH ~6 to pH ~8 and was maximal above pH 8.5 (fig. 4A). To test whether membrane-embedded carboxylic residues are involved in this pH-dependent ligand binding, the effect of DCCD and a D142C mutation of LmrP were investigated (fig. 4A). Hydrophobic DCCD completely inhibited tetracycline labeling of LmrP. A D142A mutation of LmrP caused a decreased affinity for tetracycline, but less pronounced than in the presence of DCCD. All these data are consistent with the idea that the drug- binding site is located in the membrane-embedded region of the protein and contains several carboxylic amino acid residues. Moreover, protonation/deprotonation of these membrane-embedded carboxyl residues including residue Asp^{142} seems necessary to modify drug affinity during the transport process. Indeed, when residue Asp^{142} was replaced with an alanine, tetracycline binding was still pH dependent but showed an inflection around 6.5 instead of at pH 7.5 in the WT LmrP. This shift suggests that several residues with different pKa cooperate in LmrP substrate affinity.

When a ΔpH was imposed across the membrane (ΔpH = 0.9, interior alkaline), the tetracycline labeling was increased 2.5-fold compared to the labeling observed around pH 8.5 (fig. 4B). This demonstrates for the first time that the pmf across the lipidic membrane stimulates drug binding. The fact that the hydrophilic EDAC had no effect on the pH dependency of tetracycline binding (fig. 4C) but inhibited the stimulatory effect of the pmf (fig. 4B) suggests a role for some carboxylic residues located in a hydrophilic environment in the pmf-driven stimulation. This was confirmed with mutant D128C (fig. 4B, C). Whereas its drug affinity in the absence of a ΔpH was similar to the WT protein, no stimulation occured after imposition of the ΔpH.

The affinity of mutant D68C for tetracycline was reduced even in the absence of a ΔpH (fig. 4C). This means, in addition to earlier proposals [19], that some residues located in extramembrane domains may modulate LmrP substrate affinity.

Discussion

In this study, we used the purified and liposome-reconstituted secondary MDR transporter LmrP to investigate the impact of the pmf on the structure and ligand binding of LmrP. The results demonstrate that imposition of a ΔpH across the lipidic membrane modifies globally the structure of LmrP. Indeed, the ΔpH was demonstrated to mediate a conformational change of LmrP that stimulates its drug affinity. These results provide, to the best of our knowledge, the first experimental evidence that the pmf modifies the affinity of a secondary transporter for a substrate. Mutation of carboxylic residues Asp^{68} and Asp^{128}, located in the extramembrane domain of the protein abolishes this effect. As suggested by Yu et al. [5], this pmf-mediated restructuring may be essential in other secondary MDR transporters such as AcrB.

In agreement with the data obtained from the three-dimensional crystals of ligand-bound AcrB [5], no ligand-induced (tetracycline, ethidium bromide, Hoechst 33342, verapamil, vinblastine) accessibility changes of LmrP were observed in the absence of a pmf. Notably, drug binding in the presence of the ΔpH mediates a change that increases LmrP accessibility toward the hydrophilic quencher. Several membrane-embedded residues including Asp^{142} are involved in this restructuring which may be related to the translocation of the drug binding site from a membrane-facing orientation toward an external medium-facing orientation, in favor of drug release. The fluorescence quenching was similar for all ligands used, indicating that the conformational changes required for transport are similar for drugs with different

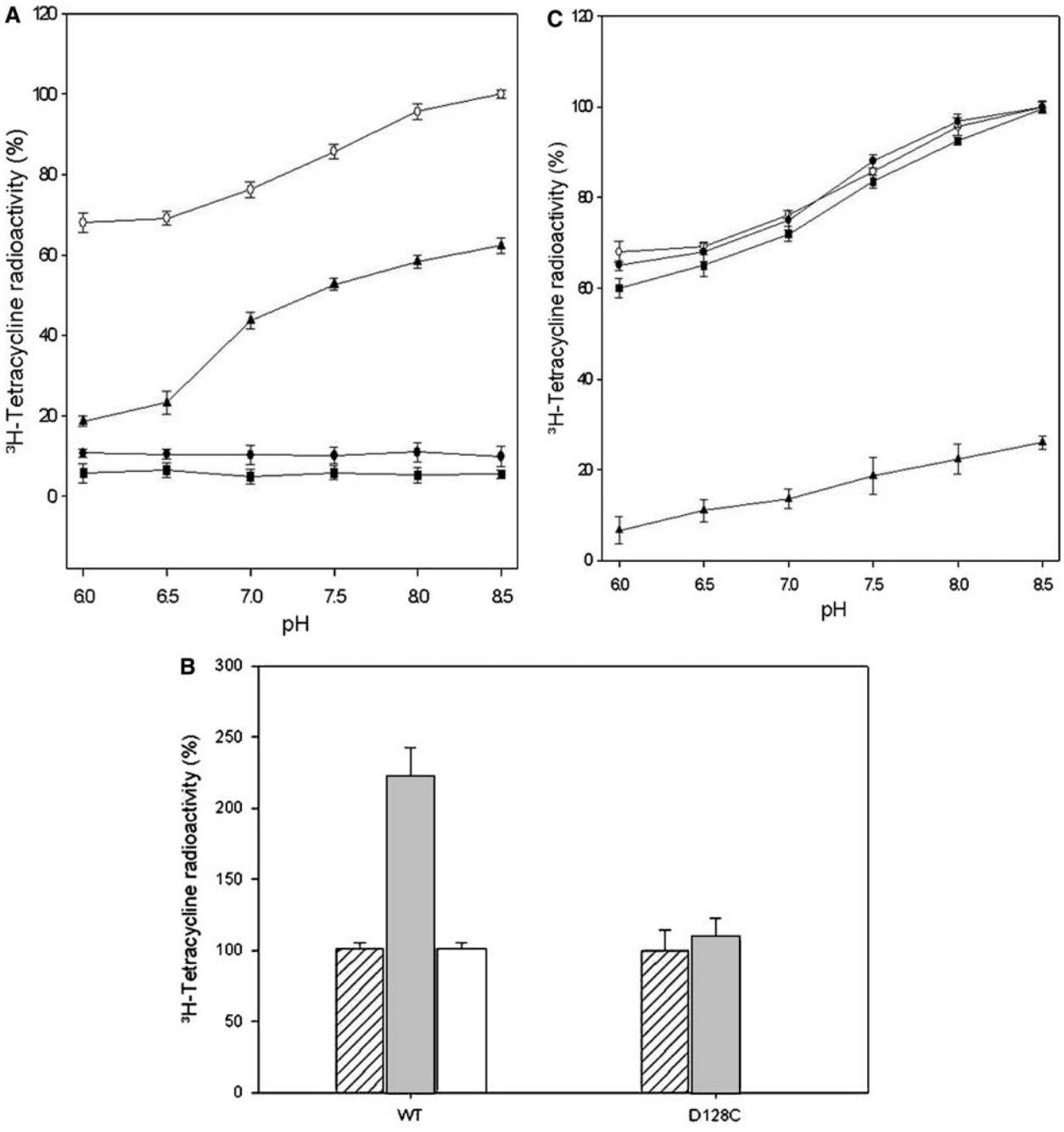

Figure 4. Effect of pH on photolabeling by [^{3}H]tetracycline of reconstituted LmrP. Reconstituted LmrP was incubated with [^{3}H]tetracycline and irradiated with UV light. The sample was run on SDS-PAGE, cut out of the gel, and radioactivity associated with the protein was determined by a Packard scintillation counter. [^{3}H]tetracycline labeling was carried out in buffers that covered a pH range from 6 to 8.5. 100 % was assigned to the maximal radioactivity detected at pH 8.5 whereas 0 % was assigned to the radioactivity detected in a portion of SDS-PAGE on the lane of the protein. (*A*) Tetracycline binding on liposomes only (■); WT LmrP-containing liposomes (○); WT LmrP-containing liposomes in the presence of 100 μM DCCD (●) and D142A LmrP-containing liposomes (▲). (*B*) Tetracycline binding on WT or D128C at pH 8.5 (hatched bars) or after imposition of a ΔpH (gray bars) and for WT after imposition of a ΔpH in the presence of 100 μM EDAC (white bars). (*C*) Tetracycline binding on WT LmrP-containing liposomes (○); WT LmrP-containing liposomes in the presence of 100 μM EDAC (●); D68C LmrP-containing liposomes (▲) and D128C LmrP-containing liposomes (■).

interaction sites with LmrP. Reorganization of the cytosolic domains due to the pmf is a prerequisite for the subsequent ligand binding-mediated restructuring. Previously, maleimide-labeling studies of cysteine mutants of LmrP demonstrated that in the absence of a pmf, the membrane-embedded Asp^{142} and Glu^{327} move from a solvent-inaccessible to a solvent-exposed environment upon interaction with substrates [19]. However, the fraction of LmrP labeled by maleimide was small under these conditions, and may indicate that observed changes are limited in the absence of a pmf. These observations, together with the data presented here, suggests that the pmf and in particular the ΔpH are not necessary for substrate binding but are crucial at least for a restructuring of LmrP that stimulates ligand binding during the transport process.

We demonstrated that tetracycline binding is pH dependent. Indeed, it increases significantly between pH 6.5 and 8.5 suggesting that deprotonation of the drug-binding site is required for ligand binding, and protonation for drug release. The inhibitory action of carbodiimides suggests that carboxylic residues located in a hydrophobic environment, probably membrane-embedded, are responsible for this pH-dependent drug affinity of LmrP. Substrate binding depending on the protonation state of membraneous carboxylic residues has also been demonstrated for the *E. coli* drug transporter EmrE [18]. After replacement of residue Asp^{142} by an alanine, tetracycline binding decreases, showing that the carboxylic residue Asp^{142} is involved in this LmrP pH-dependent drug binding. The shift of the apparent pKa of tetracycline binding to the D142A mutant from ~7.5 to ~6.5 raises the idea that several membrane-embedded carboxylic residues with different pKas cooperate to modulate LmrP affinity for drugs. A carboxylic residue located in an extramembrane loop was also demonstrated to modulate drug binding to LmrP. Namely, after mutation of residue Asp^{68} to Cys, drug binding is drastically reduced. However, it is not clear if this phenomenon results from a direct involvement of this aspartate in substrate binding. This suggests that LmrP may require a more complex mode than Emr E for coupling H^+ and drug translocation.

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