INTERACTIONS BETWEEN PHOSPHATIDYLETHANOLAMINE HEADGROUP AND LMRP, A MULTIDRUG TRANSPORTER: A CONSERVED MECHANISM FOR PROTON GRADIENT SENSING?

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In a number of cases, the function of membrane proteins appears to require the presence of specific lipid species in the bilayer. We have shown that the secondary multidrug transporter LmrP requires the presence of phosphatidylethanolamine (PE), as its replacement by phosphatidylcholine (PC) inhibits transport activity and directly affects its structure, though the underlying mechanism was unknown. Here, we show that the effect of PE on the structure and the function of LmrP is mediated by interactions between the lipid headgroup and the protein. We used methyl-PE and dimethyl-PE analogs of PE to show that only replacement of the three hydrogens by methyl moieties leads to changes in the biochemical and biophysical properties of the reconstituted protein. This suggests that LmrP does not depend on the bulk properties of the phospholipids tested but solely on the hydrogen bonding ability of the headgroup. We then show that a single point mutation in LmrP, D68C, is sufficient to recapitulate precisely every biochemical and biophysical effect observed when PE is replaced by PC, including energy transfer between the protein tryptophan residues and the lipid headgroups. We conclude that the negatively charged D68 is likely to participate in the interaction with PE, and that such interaction is required for proton gradient sensing, substrate binding and transport. As D68 belongs to a highly conserved motif in the Major Facilitator Superfamily (MFS) (1;2), this family regroups a very large number of transporters, ubiquitously found in all classes of organisms, that rely on chemosmotic ion gradients to transport their cognate substrates across the cell membrane (3). While the substrates vary greatly from one sub-family to the next, MFS transporters share a common architecture, composed of 12 transmembrane α-helices connected by extramembrane loops, as confirmed by the crystal structures of LacY (4), GlpT (5) and EmrD (6). In addition to their biological and physiological relevance, MFS transporters have proven to be very useful models to study the fundamental features of membrane proteins, and in particular how they interact with the membrane. Several studies on various MFS transporters have shown that their function depends on the lipid composition of their environment. For instance, LacY (7), GabP (8) and PheP (9) require the presence of phosphatidylethanolamine (PE) in the bilayer for proper structure and activity. Such studies illustrate that the membrane not only provides a hydrophobic environment to the embedded proteins, but that, in addition, specific lipidic species must interact with these proteins to achieve biological function (10). However, the molecular mechanisms that underlie such partnership remain often unclear. Is the protein function depending on bulk properties of the membrane bilayer or are specific interactions between the lipids and the protein necessary? In several crystal structures, lipids known to be important for function remained specifically associated to the protein, such as for yeast cytochrome bc1 complex (11) or Rb. sphaeroides photoreaction centre (12;13). However, in other cases, the bulk properties of the membrane bilayer such as fluidity were shown to be important. For instance, a clear correlation between membrane protein activity and membrane fluidity was described for calcium-
dependent ATPase (14) and the ABC transporter cdr1p (15).

In the case of LmrP, PE is essential as its replacement by PC, though another zwitterionic lipid, led to a serious alteration of structure and function (16). We wanted to investigate the mechanisms responsible for this behavior, and determine whether the bulk properties of PE provide the adequate environment to LmrP or whether interactions between the lipid headgroup and the protein are required to achieve biological function.

We assess here the role of PE over the protein function and conformation by using PE methylated species. We show that PE methylated species where one or two headgroup proton(s) are replaced with methyl moieties (methyl PE and dimethyl PE), can substitute for PE without loss of LmrP activity while replacement of the third proton abolishes all activity. Biophysical measurements show that the structural changes associated with the replacement of PE by PC are not present when we use the methyl and dimethyl intermediates, which suggests a role of the lipid headgroup and a possible interaction between PE and LmrP specific amino acids. We propose here that these residues include Asp 68, a highly conserved residue in the MFS, and that this protein-lipid interaction is a global mechanism of PE-dependence in MFS and is involved in ΔpH sensing.

Experimental Procedures

Cell culture and protein purification- The growth of L. lactis NZ9000 in M17 medium (from Difco), the overexpression using the NICE system and the purification of the His-tagged LmrP wt and mutants were performed as previously described (2;16-20).

LmrP reconstitution- We have previously shown that L. lactis membrane contains significant amount of PE (16). As the exact fraction is not established, we have used the lipid composition found in another bacteria, E. coli (70% of PE). As a control, we tested that such proportion was not limiting for LmrP activity (see Supplemental Data). Therefore the respective lipid composition of the various liposomes were: i) PE proteoliposomes: 70% dioleoyl-phosphatidylethanolamine (DOPE), 20% E. coli phosphatidylglycerol (PG), 10% E. coli cardiolipin (CL); ii) methyl PE proteoliposomes: 70% N-methyl DOPE, 20% E. coli PG, 10% E. coli CL; iii) dimethyl PE proteoliposomes: 70% N,N-dimethyl DOPE, 20% E. coli PG, 10% E. coli CL and iv) PC proteoliposomes: 70% dioleylphosphatidylcholine (DOPC), 20% E. coli PG, 10% E. coli CL. Protein reconstitution and imposition of an artificial proton gradient (ΔpH) were performed as previously described (16;21). All the phospholipids were purchased from Avanti Polar Lipids.

Hoechst 33342 transport in proteoliposomes- Hoechst 33342 transport carried out with proteoliposomes containing 10 µg wt and mutated LmrP was driven by an imposed ΔpH, as explained previously (16;21). Hoechst 33342 fluorescence was monitored on a SLM Aminco 8000 fluorimeter using excitation and emission wavelengths of 355 and 457 nm, respectively.

Tryptophan fluorescence quenching- Tryptophan fluorescence quenching experiments were carried out with hydrophilic (acylamide) quencher as described elsewhere (16;21). Data were subjected to a linear fit up to 100 mM acrylamide.

Fluorescence Resonance Energy Transfer (FRET)- 5% of dansyl-PE with respect to the total lipid mass was incorporated into proteoliposomes during the reconstitution process described above. The tryptophan-dansyl FRET was monitored on a SLM Aminco 8000 fluorimeter.

Attenuated Total Reflection-Fourier Transform Infrared spectroscopy (ATR-FTIR)- ATR-FTIR spectra were recorded at room temperature on Bruker IFS55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector at a nominal resolution of 2 cm⁻¹. The spectrophotometer was continuously purged with air dried on a FTIR purge gas generator 75 – 62 Balston (Maidstone, UK) at a flow rate of 5.8 L/min. The internal reflection element was a germanium plate (50x20x2 mm) with an aperture angle of 45°, yielding 25 internal reflections (22;23).

Secondary structure determination- Quantification of secondary structure of reconstituted LmrP based on Fourier self-deconvolution of the spectra in the amide I region was performed as previously described (22;24;25)

Orientation of the secondary structures- Determination of the orientation of secondary structures of LmrP was performed as in (24;26). Briefly, spectra were recorded with parallel and perpendicular polarized incident light with respect to the normal to the ATR plate. Polarization was
expressed as function of the dichroic ratio $R_{\text{ATR}} = \frac{A}{A^\perp}$.

Hydrogen/deuterium exchange kinetics - A sample of reconstituted LmrP was deposited on a germanium plate as described elsewhere (22,23). Sample treatment with $\text{D}_2\text{O}$-saturated $\text{N}_2$ flux, spectra acquisition, and data analysis were performed as described in (16,27,28).

Site-directed labeling of LmrP - The substituted cysteine mutants (E3C, E255C) of LmrP reconstituted in PE, methyl PE, dimethyl PE and PC proteoliposomes as well as the D68C mutant reconstituted in PE proteoliposomes were biotinylated according to the protocol used on proteoliposomes by Dowhan group (7). Briefly, proteoliposomes were incubated 20 minutes at room temperature with MPB (3-(N-maleimidylpropionyl) biocytin, Molecular Probes) added to a final concentration of 200$\mu$M from freshly-prepared stock solution to biotinylate LmrP. Where indicated, proteoliposomes were incubated 10 minutes with AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, Molecular Probes)) to block external cysteine, added from a freshly-prepared stock solution and to a final concentration of 200$\mu$M. In order to inactivate the excess of maleimides, $\beta$-mercaptoethanol was added at a final concentration of 1mM. Where indicated, proteoliposomes were disrupted by OG (with $\beta$-D-octylglucoside, Sigma) at 1.5% w/v. Removal of excess of labeling reagents as well as replacing the buffer surrounding proteoliposomes were performed by centrifuging the treated samples through Bio-spin columns (Bio-Rad). Samples were subjected to SDS-PAGE/Western Blot followed by incubation with avidin-horseradish peroxidase (Avidin-HRP, Pierce). SuperSignal West Pico chemiluminescent substrate (Pierce) was used to visualize biotinylated proteins.

RESULTS

Does PE hydrophilic moiety contribute to LmrP wt activity? The effect of PE polar head over LmrP function was investigated in PE, methyl PE, dimethyl PE and PC proteoliposomes (see “Experimental Procedures” for the detailed lipid composition of the liposomes). The positively charged Hoechst 33342 is a substrate for LmrP (21,29) which becomes highly fluorescent when inserted into lipid membranes while almost not fluorescent in aqueous medium. LmrP transport activity was assayed in PE, methyl PE, dimethyl PE and PC proteoliposomes, by measuring the variation in Hoechst 33342 fluorescence as a function of time (Fig. 1). When a $\Delta\text{pH}$ is imposed, the fluorescence of membrane-bound Hoechst 33342 decreases at the same rate in PE, methyl PE and dimethyl PE proteoliposomes indicating that Hoechst 33342 was actively being extruded out of the membrane. These data suggest that at least one free proton on PE polar head is required to sustain a normal transport activity of LmrP. As previously observed (16), the third methylation (PC) entirely abolished the Hoechst 33342 active extrusion from the membrane.

Structural characterization of LmrP in the different proteoliposomes.

In order to assess whether the inhibition of transport activity observed in PC proteoliposomes was due to structural defects, we compared the structural profiles of LmrP in various lipid environments. FTIR spectroscopy was used to determine and compare the secondary structure of LmrP in the various proteoliposomes. As shown in Figure 2, the amide I absorption peak is identical in all four liposomes. Student t-test on the spectra revealed no significant difference (at either $p<0.05$ or $p<0.01$) in the amide I region, indicating that the secondary structure of LmrP is identical in either PE, methyl PE, dimethyl PE or PC proteoliposomes. This secondary structure was estimated after deconvolution to be composed of 69% $\alpha$, 7% $\beta$ and 24% coiled, which is in good agreement with the secondary structures seen in the crystal structures of LacY, GlpT and EmrD. Using polarized ATR-FTIR spectroscopy we compared the average helix orientation in the bilayer for PE and PC-containing proteoliposomes. Quantitative evaluation of the helix mean tilt requires the evaluation of the dichroic ratio $R_{\text{ATR}} = \frac{A}{A^\perp}$ for amide $\nu(\text{C}=\text{O})$ and $R_{\text{iso}}$ which is the dichroic ratio measured for a transition dipole either spatially disordered or oriented at the magic angle (30). The derived dichroic ratio can detect orientation changes as small as 8% (24). As described elsewhere (30), lipid ester $\nu(\text{C}=\text{O})$ was used to determine $R_{\text{iso}}$. Dichroism analysis yielded $R_{\text{iso}} = 1.6$ for wt LmrP in PE and PC proteoliposomes (Table 1). Furthermore, $R_{\text{ATR}}$ measured for wt in...
PE proteoliposomes was identical to that of wt in PC proteoliposomes. In order to analyze solely the orientation of the transmembrane bundle, this ratio was calculated on the helical contribution obtained from the deconvolution of the FTIR spectra (maximum intensity at 1652 cm\(^{-1}\)). Finally the topology of LmrP insertion (inside-out vs. right side-out) was measured in the different proteoliposomes using the substituted cysteine accessibility method (SCAM) to determine whether a cysteine residue is entrapped inside or located on the outside of the proteoliposome. Two LmrP single cysteine mutants that display wt-like activities (18) were used: E3C, located in the N-terminal tail on the cytoplasmic side and E255C located in the fourth extramembrane loop (ECL4) on the extracellular side (Fig. 3). These single cysteine mutants were each reconstituted into PE, methyl PE, dimethyl PE and PC proteoliposomes. The orientation of the N-terminal tail and ECL4 in proteoliposomes was studied by analyzing the accessibility of each single cysteine residue in these domains to MPB 3-(N-maleimidylpropionyl) biocytin, Molecular Probes), a thiol specific reagent as described by Dowhan and co-workers (7). The reactivity of the cysteine in N-terminal tail (E3C) and ECL4 (E255C) in proteoliposomes is shown in Figure 4. In the four proteoliposomes (PE, methyl PE, dimethyl PE and PC) E3C mutant was labeled by MPB, suggesting that the N-terminal tail is located on the outside of proteoliposomes. To ensure that no cysteines were trapped inside the proteoliposomes, samples were pre-treated with the membrane impermeable blocking reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Molecular Probes) to saturate all the cysteines on the outside, and then permeabilized with β-D-octylglucoside (OG, Sigma) and treated with MBP. For the E3C mutant, no MBP labeling was observed (Fig. 4), suggesting that no LmrP molecule was reconstituted with the N-terminus inside the proteoliposomes. In contrast, mutant E255C was not labeled after MPB treatment and was only labeled when the proteoliposomes were solubilized with OG, supporting that the cysteine is only located on the inside of the proteoliposomes. The results indicate that in the four proteoliposomes, LmrP is inserted with the N-terminal tail on the outside and ECL4 on the inside, suggesting an inside-out orientation of LmrP relative to the situation in the bacterial cell. Inside-out orientation in PE and PC proteoliposomes has also been demonstrated for LacY (7).

From these experiments we conclude that the lack of transport in PC proteoliposomes is not a consequence of inadequate LmrP topology or insertion in the bilayer.

Does PE polar head affect LmrP accessibility to the aqueous medium?

-Fluorescence quenching. Conformational diversity can be probed using tryptophan fluorescence quenching. Five out of seven LmrP tryptophan residues are predicted to be located in the extramembrane domains (21;29). Since LmrP is inserted in inside-out fashion, the Trp residues of LmrP whose fluorescence will be quenched by acrylamide are W5 and W285, located in the N-terminal tail and the intracellular loop 4 (ICL4) respectively (Fig. 3). The accessibility of the two Trp residues to the aqueous medium would reflect the accessibility of these two cytosolic domains. The solvent exposure of the Trp side chains was determined by monitoring the fluorescence intensity in the presence of increasing concentrations (0-100 mM) of the hydrophilic quencher acrylamide (31;32). Fig. 5 shows the Stern-Volmer plots of Trp fluorescence quenching by acrylamide for LmrP reconstituted in PE, methyl PE, dimethyl PE and PC proteoliposomes. After a proton gradient was imposed on PE proteoliposomes, the Trp accessibility was increased, indicating a reorganization of the cytosolic domains upon ΔpH imposition. As shown in Fig. 5, a similar behavior was observed in PE, methyl PE and dimethyl PE proteoliposomes. Surprisingly, imposing a proton gradient on PC proteoliposomes did not lead to any LmrP conformational change in the cytosolic region. Therefore, the partial modification of the PE headgroup does not appear to modify the structural properties of LmrP while removing the last headgroup hydrogen strongly affects Trp exposure. This suggests that the PE headgroup and LmrP interact directly.

-IR spectroscopy. At constant pH and temperature, the rate at which hydrogen and deuterium exchange reflects the solvent accessibility of the NH amide groups of the protein, which in turn is related to the tertiary structure of the protein and to the stability...
of secondary structure elements (33). H/D exchange was monitored on LmrP reconstituted in PE, methyl PE, dimethyl PE and PC proteoliposomes. LmrP amide proton exchange was followed by monitoring the amide II absorption peak (δ(N-H), maximum at 1544 cm⁻¹) decrease as function of time of exposure to a D₂O-saturated N₂ flow (see “Experimental Procedures”). H/D exchange rate was quantified by monitoring the evolution of the amide II area for each sample between 0 and 100% and reported in Fig. 6. After a two-hour deuteration, 55% of LmrP N-H had been exchanged in PE proteoliposomes. That rate was identical to that of methyl PE and dimethyl PE proteoliposomes. The rate of H/D exchange was higher in PC proteoliposomes as 70% of the amide N-H had been exchanged, suggesting that PC favors a lower-stability state of LmrP. With 60% of LmrP included in the transmembrane bundle (21), this indicates that the change in solvent exposure in PC liposomes must involve to some extent the TM helices of LmrP.

Our experiments indicate that a hydrogen-bonding capacity of the lipid headgroup must be preserved in order to observe activity of the reconstituted LmrP (see “Discussion”). Of interest, we also observed normal transport activity when PE is replaced by phosphatidylserine (see Supplemental Data), further supporting that the hydrogen bonding capacity of the lipid headgroup is the key feature of lipids allowing LmrP activity.

While such hydrogen bonds could indirectly influence the structure of LmrP, a simpler explanation would involve a direct interaction between LmrP and PE headgroup. Following this working hypothesis we wanted to test the role of strong hydrogen bond acceptors in the protein (Glu or Asp) in the interaction with PE.

Cysteine-scanning mutagenesis has been previously performed on all acidic residues of LmrP (18). Mutants D68C, D128C, D235C and E327C have been demonstrated to lose their ability to transport substrates from L. lactis cells. In addition, D68C and D128C do not show proton gradient-induced conformational changes (21) while mutant D68C has completely lost the ability to bind [³H]-Tetracycline. Remarkably, once LmrP is reconstituted in PE-deficient liposomes, the transporter is also unable to bind [³H]-Tetracycline (data not shown). In summary, mutant D68C reconstituted in PE-containing liposomes recapitulates every phenotype previously established for the wt LmrP when reconstituted in PE-deficient liposomes, suggesting that this acidic residue might be directly involved in the interaction between LmrP and PE. We therefore tested the biophysical correlation between the loss of PE and the replacement of D68 to further probe its possible involvement in an interaction with the lipid headgroup.

**D68 mutation and PE replacement with PC lead to indistinguishable structural states.**

The D68C mutant was reconstituted in PE-liposomes and tested for structural integrity. Cysteine labeling shows, as for the wt, that the protein is solely oriented in an inside-out fashion (Figure 4). Polarized ATR-FTIR dichroic ratio (Table 1) was identical to that of wt LmrP, indicating that the protein topology is not affected by the mutation.

In order to compare the behavior of D68C mutant in PE liposomes to that of LmrP wt in PC liposomes, we first measured the protein-membrane interaction by determining the fluorescence resonance energy transfer (FRET) between the tryptophan residues of LmrP and fluorophores attached to lipids. We used dansyl-PE where the dansyl fluorophore is located within the head group region of the vesicle lipid bilayer. The transfer of fluorescence energy from tryptophan to dansyl-PE is reported for wt LmrP in PE, wt in PC and D68C in PE proteoliposomes in Fig. 7. Transfer was more than twice higher in wt-PE proteoliposomes than in D68C-PE proteoliposomes, showing that replacing one amino acid affects the average position of the tryptophan residues. Moreover, FRET of D68C mutant reconstituted in PE proteoliposomes was identical to that of wt reconstituted in PC proteoliposomes, indicating a similar change in the location and dynamics of tryptophan in both cases.

The effect of the D68C mutation on the protein stability was measured by deuterium exchange on proteoliposomes containing PE and compared to that of the wt protein reconstituted in PC-substituted liposomes. Fig. 6 shows the percentage of non exchanged N-H of LmrP D68C reconstituted in PE liposomes as function of time.
After 2 hours of deuteration, the rate of exchange was similar for LmrP D68C in PE proteoliposomes and LmrP wt in PC proteoliposomes. As both changes affect the stability of LmrP in a similar manner, this further supports the hypothesis that D68 is involved in the interaction between LmrP and PE.

DISCUSSION

Specific vs. nonspecific interaction with PE
Understanding the nature of the interaction between PE and the multidrug transporter LmrP is key to understand the molecular mechanism of action of such transporters. We asked here whether PE provides particular physico-chemical characteristics to the bilayer necessary for LmrP activity, or whether it plays a more specific role by interacting directly with the transporter. The functional importance of PE has been demonstrated for several other MFS. In the case of LacY, Chen and Wilson (34) have shown that PE is necessary for transport activity, whereas mono- and dimethylation reduced transport activity. They also showed that, to some extent, PE could be replaced by phosphatidylserine, suggesting that hydrogen bonding ability might be required. Dowhan and coworkers have demonstrated a crucial role of PE for different MFS, such as PheP (9), GabP (8) and LacY (7). For a number of other membrane proteins, phosphatidylethanolamine has been shown to be important (for review, see (10)). In the case of rhodopsin, it was suggested that the increased activity seen when PE is present is due to the bulk properties of the lipid (i.e. favoring of the hexagonal HII phase). However, in other cases, a more specific role of the headgroup was proposed, where the protein side chains would directly interact with the NH3+ moiety. For the mechanosensitive channel Mscl, interactions involving the PE polar headgroup region of the bilayer could be responsible for the tension sensing (10;35).

It is well known that replacing the primary ammonium in PE by a tertiary ammonium changes more than just the polarity of the headgroup as a number of properties of the membranes are affected. One can therefore ask wether the loss of LmrP activity observed in PC-proteoliposomes is due to such modification of the membrane or if the headgroup itself is more directly involved in the function of the transporter.

The use of PE analogs which differ solely in the methylation state of the ethanolamine headgroup offers a way to address this issue as many of such physico-chemical properties have been determined for the analogs as well.

For LmrP activity, the lipid environment in the PC-liposomes is radically different than the ones found in the three other liposomes tested, as one or two methylations still permit wt-like function and structure but replacing all three hydrogens by methyl moieties completely inhibits transport and strongly modifies the structural features of the reconstituted protein.

On the other hand, many of the physico-chemical properties of the two methylation intermediates are evenly distributed between what is observed for PE and for PC. In other words (and in contrast to what is observed for LmrP structure and activity) methyl- and dimethyl-PE do not segregate with PE when it comes to bulk properties. For example, the phase transition temperatures of the four lipids determined by differential scanning calorimetry were reported as -5.5°C (DOPE), -7.5°C (methyl DOPE), -12.3°C (dimethyl DOPE) and -17.2°C (DOPC) respectively (36). Geometrically speaking a similar trend is present, with intrinsic curvature values -0.48 nm⁻¹, -0.35 nm⁻¹, -0.23nm⁻¹ and -0.11nm⁻¹ for DOPE, methyl DOPE, dimethyl DOPE and DOPC respectively (37). In addition, it is known that, like DOPC, methyl DOPE and dimethyl DOPE form lamellar phases while DOPE forms hexagonal phases. Clearly, there is no obvious correlation between these bulk physico-chemical properties of the lipid species used for reconstitution and LmrP activity.

NMR studies have shown that methylating just one of PE hydrogens leads to a strong increase in hydration: methyl- and dimethyl-PE show hydration states similar to that of PC (38). In contrast, as measured by the order parameter of the amine group, mono-or dimethylation modify only moderately the hydrogen-bonding ability of the headgroup while PC is strongly affected. Therefore, the deleterious phenotype observed when replacing PE by PC could arise from the sole
removal of the hydrogen-bonding capability of the headgroup.

From their work on LacY, Dowhan and co-workers concluded that removing PE leads to a topological change in the protein that would be responsible for the lack of activity. Importantly, these authors showed that the topology of LacY is identical in proteoliposomes containing either PE or PC, albeit PC-containing liposomes showed no transport activity for the reconstituted protein (7). We have measured the polarized ATR-FTIR dichroic ratio $R_{\text{ATR}}$ to assess the protein topology, and observed that LmrP shows a dichroic ratio of 1.82 ± 0.02 in both PE and PC proteoliposomes. ATR-FTIR is particularly sensitive to conformational changes and a topological reorganization such as the one suggested by Dowhan and coworkers (that requires the reorientation of at least one transmembrane helix) would, according to (24), lead to a dichroic ratio $R_{\text{ATR}}$ of 1.75 or less. Thus, and considering that the secondary structure and the insertion of LmrP appear identical in both types of liposomes (Fig. 4), we do not believe that the effects on LmrP observed here when PE is replaced by PC are due to structural or topological misbehaving of the inserted protein.

Therefore, at this stage, we conclude that the structural and functional impairment observed when going from PE to PC liposomes are not due to insertion defect of LmrP or modification of the bulk properties of the membrane or headgroup hydration but -while we cannot exclude a combination of effects- are likely the consequence of changes in the hydrogen bonding capability in the lipid headgroup. Such hydrogen bonds would be required, in either a direct or indirect manner, for proper structuration of the transporter (as measured for H/D exchange), proton gradient sensing (as measured by Trp fluorescence quenching), binding and transport.

Residue D68 of LmrP belongs to the highly conserved G-X-X-X-D-R/K-X-G motif of the first intracellular loop (ICL1) linking TM2 and TM3 (20). This motif is remarkably preserved throughout the Major Facilitator Superfamily, despite sequence variation in all other regions. Moreover, the structural conservation of this motif is striking. It should be stressed that the three MFS transporters crystallized so far, LacY, GipT and EmrD, share at most 20 percent of overall sequence identity. There are all organized in a bundle of 12 transmembrane helices, adopting grossly the same structure, but each protein has evolved local structural characteristics and significant deviations are observed. However, when we superimpose the cytoplasmic halves of TM2 and TM3 of the three structures we observe that this region is well conserved structurally. More specifically, the G-X-X-D-R/K part of the known motif is remarkably similar in the three proteins, with both the Asp and the Arg/Lys well aligned (Fig. 8).

A series of studies have tested the role of D68 (or its equivalent) in several MFS transporters. Mutagenesis analysis demonstrated that this Asp is crucial for the transport activity (39-42). But as D68 is conserved among transporters that recognize widely different substrates, it was ruled out that this residue is involved in substrate recognition. In fact, in addition to the strong conservation of D68 throughout the MFS family, its cytoplasmic location (see Fig. 8) also indicates that it is not a binding site for hydrophobic substrates (as those transported by LmrP). Instead, various studies suggested that it plays a role in
facilitating conformational changes associated with substrate transport (42) in agreement with the results from this work.

We note that a number of MFS transporters do not harbor an Asp in ICL1. For instance the family of sodium-dependent phosphate transporters, which depend on sodium gradient to import phosphate in the kidney, has lost the D68 equivalent. Another important example is the family of organic anion transporters, that move organic anions across the basolateral membrane of the kidney into the proximal tubule through an anion/dicarboxylate mechanism (43). An Asp is also lacking in ICL1 of most glucose facilitated transporters, which are known to transport glucose in an energy-independent manner (passive transport) (44). In these various cases, substrate transport is not dependent on a proton gradient, which fits the proposed hypothesis that D68 is necessary for pH gradient sensing. However, the large MFS family is only partly characterized, and a more thorough analysis (that goes beyond the scope of this paper) would be required to determine if transporters that have lost the sensitivity to proton gradient are all devoid of Asp in ICL1 and vice versa.

**Does gradient-sensing depend on an interaction between D68 and PE?**

We have previously shown (21) that LmrP is conformationally sensitive to the proton gradient: imposing a pH gradient leads to a significant conformational rearrangement, even in the absence of substrate (which would suggest little or no proton transport by LmrP). This means that LmrP is able to structurally “sense” the gradient. This conformational sensitivity can be uncoupled from proton translocation itself as mutation of D142, believed to be involved in proton translocation (45), does not affect the pH gradient-induced conformational change (21). On the other hand, this conformational change is absent in PC-liposomes, indicating that, although all the acidic residues are present, the protein becomes unable to sense the gradient. We proposed here that the interaction between LmrP and PE is, at least in part, mediated by D68. As the chemical features of an aspartate are matching the requirements of the amine headgroup of PE, it is tempting to speculate that D68 could be interacting with the lipid headgroup. Direct interactions between acidic side chains and the amine group of PE molecules have been observed in crystals, such as in the structure of the cytochrome bc1 (11;46). If present in the system studied here, such direct contact would explain why removing the Asp side chain or replacing PE by PC lead to indistinguishable phenotypes. A key implication of such hypothesis would be that this interaction is necessary for the protein to be sensitive to the proton gradient. Note that, in fact, the model does not rule out an indirect D68-PE interaction, mediated by a third molecular party.

It should be stressed that most studies on MFS transporters have focused on the effect of lipid changes or mutations on the transporter’s activity, either binding or transport. Our studies on LmrP using Trp fluorescence have shown that the proton gradient induces a conformational change that is lost in the absence of PE or D68. Therefore one could hypothesize that a D68-PE interaction is involved in the sensitization of the protein to the pH gradient. Considering that D68 is highly conserved and that PE-dependence has been shown for a number of MFS proteins, this hypothesis would apply to many of these gradient-sensing transporters.
REFERENCES


FOOTNOTES

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ABBREVIATION LIST

PE, phosphatidylethanolamine; PC, phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleylphosphatidylcholine, PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine, MPB (3-((N-maleimidylpropionyl) biocytin; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; OG, β-D-octylglucoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MFS, major facilitator superfamily; FRET, fluorescence resonance energy transfer; ATR-FTIR, attenuated total reflection-Fourier transform infrared spectroscopy; TM, transmembrane domain; ICL, intracellular loop; ECL, extracellular loop.

TABLE TITLES

Table 1. Dichroic ratio $R^{ATR}$ of the alpha helix band of LmrP wt reconstituted in PE or PC proteoliposomes and D68C in PE proteoliposomes.
FIGURE LEGENDS

Fig. 1. Hoechst 33342 transport by LmrP reconstituted into PE, methyl PE, dimethyl PE proteoliposomes. Proteoliposomes were diluted 100-fold in the buffer used for reconstitution. A ΔpH was imposed as described in “Experimental Procedures”. After 50 seconds of spectra acquisition, 1µM Hoechst 33342 was added. Fluorescence intensity after addition of Hoechst 33342 was normalized to 100%, and was monitored for 300 s. The results shown are typical of 3 independent experiments.

Fig. 2. ATR-FTIR amide I absorption band of reconstituted LmrP in PE, methyl PE and dimethyl PE and PC proteoliposomes and D68C in PE proteoliposomes.

Fig. 3. Helical net representation of LmrP. Helix ends were deduced from the sequence alignment of LmrP with the published structure of EmrD (6). Residues Glu 3, Trp 5, Asp 68, Asp 255 and Trp 285 are highlighted.

Fig. 4. Determination of the orientation of the N-terminal tail and the ECL4 loop of LmrP in PE, methyl PE, dimethyl PE and PC proteoliposomes or ICL2 of D68C in PE proteoliposomes. Single cysteine in the N-terminal tail, ECL4 or in ICL2 were treated with MPB directly, after pre-treatment with AMS or after solubilization with OG. Samples were subjected to SDS-PAGE. Biotinylation with MPB was detected after transfer to nitrocellulose sheet using avidin-HRP. The results shown are typical of 3 independent experiments. Note that a minor degradation product is sometimes observed below the full length LmrP band.

Fig. 5. Trp quenching of LmrP reconstituted in PE, methyl PE, dimethyl PE and PC proteoliposomes by acrylamide: Stern-Volmer plots. F is the measured fluorescence intensity and F0 is the fluorescence intensity in the absence of acrylamide. Error bars, shown when they are larger than the symbol size indicate standard deviation. The points are the average of at least three independent experiments.

Fig. 6. Percentage of non exchanged amide protons reported as function of the deuteration time for LmrP wt reconstituted into PE (○), methyl PE (×), dimethyl PE (△) and PC (○, gray) proteoliposomes and for D68C reconstituted into PE proteoliposomes (×, gray). The percentage of deuteration was estimated from the amide II surface evolution as described under “Experimental Procedures”. Each curve represents the average of three independent experiments. Error bars, shown when larger than point symbol, represent the standard deviation from the average.

Fig. 7. Fluorescence resonance energy transfer from the protein tryptophans to dansyl-PE in LmrP wt-PE, wt-PC and D68-PE proteoliposomes. Wt-PE proteoliposomes containing no dansyl-PE were used as control. The results shown are typical of 3 independent measurements.

Fig. 8. Ribbon representation of the G-X-X-X-D-R/K-X-G motif in the structure of LacY, EmrD, GlpT (2CFG, 2GPP, 1PWY respectively). The cytoplasmic halves of TM2 and TM3 were superimposed. The side chain of Asp, Lys and Arg are shown in solid stick. The glycine Cα’s are shown as balls.
Table 1

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<th>$R^{iso}$</th>
<th>$R^{ATR}$</th>
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<tr>
<td>WT-PE</td>
<td>1.64 ± 0.05</td>
<td>1.82 ± 0.02</td>
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<tr>
<td>WT-PC</td>
<td>1.59 ± 0.05</td>
<td>1.82 ± 0.02</td>
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<tr>
<td>D68-PE</td>
<td>1.61 ± 0.04</td>
<td>1.83 ± 0.02</td>
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Figure 4

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<td>D68C</td>
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Figure 5

![Graph showing the relationship between F0/F and [Acrylamide] (M) with different conditions: PE + ΔpH, methyl PE + ΔpH, dimethyl PE + ΔpH, PC + ΔpH, PE − ΔpH, methyl PE − ΔpH, dimethyl PE − ΔpH, PC − ΔpH.](image-url)
Figure 8
Interactions between phosphatidylethanolamine headgroup and LMRP, a multidrug transporter: A conserved mechanism for proton gradient sensing?
Pierre Hakizimana, Matthieu Masureel, Bénédicte Gbaguidi, Jean-Marie Ruysschaert and Cedric Govaerts

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