Lipid Composition Regulates the Orientation of Transmembrane Helices in HorA, an ABC Multidrug Transporter*§

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ATP-binding cassette (ABC) transporters constitute a large class of molecular pumps whose central role in chemotherapy resistance has highlighted their clinical relevance. We investigated whether the lipid composition of the membrane affects the function and structure of HorA, a bacterial ABC multidrug transporter. When the transporter was reconstituted in a bilayer where phosphatidylethanolamine (PE), the main lipid of the bacterial membrane, was replaced with phosphatidylcholine (PC), ATP hydrolysis and substrate transport became uncoupled. Although ATPase activity was maintained, HorA lost its ability to extrude the prototypical substrate Hoechst33342. Attenuated Total Reflection–Fourier Transform Infrared spectroscopy (ATR-FTIR) revealed that, although the secondary structure of the protein was unaffected, the orientation of the transmembrane helices (TM) was modified by the change in lipid composition. The orientation of the backbone carbonyls indicated that the helices opened wider in PE versus PC-containing liposomes, with 10 degrees difference. This was supported by hydrogen/deuterium exchange studies showing increased protection of the backbone from the solvent in PC-containing liposomes. Electron Paramagnetic Resonance was used to further probe the structural change. In the PC-containing liposomes we observed increased mobility of the spin label in TM4, along with increased exposure to molecular oxygen, used as a hydrophobic quencher. This indicates that the lipid change induced modification of the orientation of TM4, exposing Cys-180 to the lipid phase. The lipid composition of the bilayer thus modulates the structure of HorA, and in turn its ability to extrude its substrates.

Over the last thirty years, our vision of the biological membrane has evolved from the fluid mosaic model (1) to that of a remarkably complex system where a myriad of molecules are tightly organized along the membrane plane to properly interact and achieve numerous biological functions. Precise interplay between lipids and proteins must occur to achieve biological function, and membrane proteins have evolved specific sequence motifs to adapt to their environment. Studies have begun to evidence how proteins and lipids interact. It emerges notably that membrane proteins are surrounded by a shell of slow-moving lipids (annular lipids) distinguishable from the bulk phase. Other lipids (non-annular lipids) can achieve tight and specific interactions with a protein and act as cofactors essential to protein function (2, 3). The lipid composition of the membrane can have significant effects on, and even regulate, membrane protein function. In a number of cases, protein function is proposed to be influenced by the bulk physico-chemical properties of the membrane, which in turn are strictly determined by the exact lipid composition of the bilayer (2–4). Such properties include hydrophobic thickness (4), phase transition (5), curvature (6), and lateral pressure (3). It has also been proposed that a specific lipid might act as a molecular chaperone required for proper folding and topology of the membrane protein (7).

We have recently proposed that protein function may require specific interactions between protein motifs and the lipid headgroup (8). Specifically, we postulated that the polar moiety of phosphatidylethanolamine (PE) interacts with one or several sidechains of the secondary transporter LmrP, this interaction being required to allow sensing of the proton gradient, the energy source for transport. This suggests that the protein has evolved one or more specific PE-binding motifs and that, although abundant in the bacterial membrane, PE acts as a cofactor playing a structural and functional role akin to that of structural water molecules in soluble proteins. It also suggests that such functional adaptation should be found in other types of membrane proteins.

The ATP-binding cassette (ABC) transporters are of particular interest because of their central role in multidrug resistance (MDR). In bacteria, numerous ABC exporters are responsible for active extrusion of antibiotics. In humans, transporters such as MDR1 (also known as P-glycoprotein) participate in tumor resistance to chemical treatment by removing the drug from cancer cells.

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§ The abbreviations used are: PE, phosphatidylethanolamine; ABC, ATP-binding cassette; ATR-FTIR, attenuated total reflection-Fourier transform infrared spectroscopy; CL, cardiolipin; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; ECL, extracellular loop; EPR, electron paramagnetic resonance; ICL, intracellular loop; NBD, nucleotide-binding domain; PC, phosphatidylcholine; PG, phosphatidylglycerol; TMD, transmembrane domain; MDR, multidrug resistance.
Despite their remarkably diverse spectra, ABC transporters share a common architecture consisting of 2 transmembrane domains (TMDs) and 2 nucleotide-binding domains (NBDs). These domains can be linked within one polypeptide (as for MDR1), encoded by different genes, or produced by homodimerization of two TMD-NBD half-transporters (typical of bacterial transporters). The TMDs are responsible for substrate selectivity and transport, whereas ATP hydrolysis in the NBDs provides the energy input.

In this work, we have tested the role of PE on the structure and function of HorA, an ABC multidrug exporter of Lactobacillus brevis. We show that replacing PE with phosphatidylcholine (PC) causes concomitant structural and functional changes. Although the protein is still able to hydrolyze ATP, it has lost the ability the transport its substrate. Biophysical measurements demonstrate that the change in lipid environment affects the orientation of the TM helices.

**EXPERIMENTAL PROCEDURES**

**Bacterial Culture**

HorA was expressed in Lactococcus lactis subsp. lactis strain NZ9000 from the nisin-controlled gene expression (NICE) vector pNZHHorA, containing a sequence encoding an N-terminal His6 tag (9). Cells were grown at 30 °C to mid exponential phase (OD690 nm ~0.6) in M17 medium (Oxoid) supplemented with 5 μg/ml chloramphenicol and 0.5% (w/v) glucose. Expression was induced for 2 h with nisin A (10 ng/ml) before the cells were harvested.

**Membrane Vesicles**

The peptidoglycan cell wall was digested by incubation for 1 h at 30 °C with 10 mg/ml lysozyme (Sigma). DNase (100 μg/ml) and 10 mM MgSO4 were then added. After a 10-min incubation, the cells were lysed by three passages through a high-pressure homogenizer (Avestin) at 20,000 psi. Cell debris was removed by three consecutive centrifugation steps at 16,000 g. The resulting supernatant was further incubated at 4 °C for 30 min. Detergents were removed by 3 consecutive incubations at 4 °C with 80 mg of SM2 Bio-beads (Bio-Rad). Finally the proteoliposomes were collected by overnight centrifugation at 105,000 g, then washed with 50 mM Hepes pH 7.4, 75 mM NaCl.

**ATPase Assay**

The malachite green assay (10) was used to determine the specific ATPase activity of HorA by measuring the release of inorganic phosphate. Briefly, a protein sample (typically 10 μg) was incubated at 37 °C with 2 mM ATP in a final volume of 200 μl. At different times (0, 10, 15, 20, and 25 min.), 25-μl aliquots were transferred into 175 μl of 10 mM sulfuric acid to stop the reaction. Subsequently, 50 μl of fresh malachite green/molybdate solution was added, and the absorbance at 650 nm was measured after incubation for 10–15 min. The malachite green/molybdate solution used for staining was freshly prepared by mixing malachite green solutions 1 (0.122% (w/v) malachite green, 20% (v/v) sulfuric acid), 2 (7.5% (w/v) ammonium molybdate), and 3 (11% (v/v) Tween 20) at a ratio of 50:12.5:1.

**Transport Assay**

Hoechst 33342 was added to HorA proteoliposomes (~15 μg of protein) at 1 μM final concentration. Transport was initiated by addition of ATP at 2 μM final concentration. Fluorescence was monitored at 37 °C with an SLM Aminco 8000 fluorimeter (excitation wavelength: 355 nm; emission wavelength: 457 nm). In controls, the proteoliposome suspension was preincubated in the presence of 4 mM vanadate before addition of ATP. Curves were corrected for the contribution of the ATP-containing buffer.

**Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)**

ATR-FTIR spectra were recorded at room temperature with 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 internal reflection element (IRE) was a germanium plate (50 × 20 × 2 mm) with an aperture angle of 45°, yielding 25 internal reflections (11). The spectrophotometer was continuously purged with air dried on a 75–62 Balston FTIR purge gas generator (Maidstone, England) at a flow rate of 5.8 liters/min.

**Sample Preparation**—Thin films of oriented multilayers were deposited on the germanium plate and dried under a stream of nitrogen (12). The ATR plate was then sealed in a universal sample holder. The sample contained 30 μg of reconstituted HorA.
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Secondary Structure Determination—Quantification of secondary structure of reconstituted HorA based on Fourier self-deconvolution of the spectra in the amide I region (1600–1700 cm\(^{-1}\)) was performed as previously described (13).

Molecular Orientation Determination—Molecular orientations were determined by infrared ATR spectroscopy as previously described (11, 14). Spectra were recorded with the incident light polarized parallel and perpendicular to the germanium plate. Dichroic spectra were computed by subtracting the parallel-polarized from the perpendicular-polarized spectrum. Correlation for differences in evanescent field intensity for each polarization was done by normalizing the spectrum with respect to the area of the lipid ester band at 1740 cm\(^{-1}\), estimated to be equal in both directions (14). An upward deviation in the dichroic spectrum indicates a dipole having preferentially a near-normal orientation with respect to the ATR plate. A downward deviation on the dichroic spectrum, conversely, indicates a dipole oriented closer to the plane of the ATR plate (11). The dichroic ratio \(R_{\text{ATR}}\) is defined as the ratio of the amide I area recorded for perpendicular polarization (A\(^\perp\)) to that recorded for parallel polarization (A\(^\parallel\)) in Equation 1.

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R_{\text{ATR}} = A^\perp / A^\parallel \quad \text{(Eq. 1)}
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To obtain R\(^{\alpha, \text{hel}}\), i.e. the dichroic ratio associated with the \(\alpha\)-helical content of the protein, the parallel and perpendicular polarized spectra were subjected to Fourier self-deconvolution and curve fitting (13). The dichroic ratio R\(^{\alpha, \text{hel}}\) was then computed by dividing the area associated with the \(\alpha\)-helices in the perpendicular spectrum by the area of the \(\alpha\)-helices in the parallel spectrum.

In ATR, the dichroic ratio for an isotropic sample (R\(^{\text{iso}}\)) is different from unity and computed on the basis of the area of the lipid ester band (1762–1700 cm\(^{-1}\)). The mean orientation of a molecular axis with respect to the perpendicular of the ATR plate can be estimated from the dichroic ratio associated with this chemical bond, as described previously (for a review, see Refs. 13, 14).

Deuteration Kinetics—Films containing 30 µg of reconstituted HorA were prepared on a germanium plate as described above. Deuteration was initiated under a D\(_2\)O-saturated N\(_2\) flux, at a flow rate of 100 ml/min. For each kinetic time point, 24 scans were recorded and averaged at 4 cm\(^{-1}\) resolution. All spectra were corrected for atmospheric water absorption. The area corresponding to amide II, characteristic of the δ(N-H) vibration, was obtained by integration between 1596 and 1502 cm\(^{-1}\). For each spectrum, this area was divided by the corresponding lipid \(\nu(C = O)\) area to take into account swelling of the sample layer at the beginning of deuteration (15).

Electron Paramagnetic Resonance (EPR) Spectroscopy

CW-EPR spectra of spin-labeled HorA in DOPC and DOPE liposomes were collected with a Varian E-109 spectrometer equipped with a loop-gap resonator. Samples were loaded into the glass capillary tubes. The microwave power was 2 milliwatt incident, the modulation amplitude was 1.6 G, and the scan width used was 160 G. Power saturation experiments were carried out on a Bruker Elexsys E500 spectrometer at room temperature. Spin-labeled HorA samples were loaded into a gas-permeable TPX capillary tube, and measurements were carried out under nitrogen and in the presence of 20% oxygen or 50 mM Ni(II)ethylenediaminediacetic acid (NiEDDA). The data were analyzed to obtain the P\(_{1/2}\) parameter. The EPR accessibility parameter II was calculated as previously described (16, 17).

RESULTS

Protein Expression, Purification, and Reconstitution—HorA was expressed in _L. lactis_ as previously described (9). As described under “Experimental Procedures”, the protein was solubilized in Fos-choline-16, purified by affinity chromatography, and reconstituted in liposomes containing either 67% DOPE or 67% DOPC. For simplicity sake, these two types of proteoliposomes are henceforth, respectively, called “PE proteoliposomes” and “PC proteoliposomes.”

The composition of the PE liposomes matched that of the _E. coli_ lipids typically used for reconstituting bacterial transporters and in which HorA shows both ATPase activity (about 150 nM·mg\(^{-1}\) min\(^{-1}\)) and substrate transport (data not shown). PE and PC proteoliposomes showed similar efficiency of reconstitution, final lipid:protein ratio, migration in a sucrose gradient, and average size (data not shown). In both PE and PC proteoliposomes, HorA adopts essentially (>80%) an inside-out orientation (supplemental Fig. S1).

PE Is Required for Substrate Transport—As shown in Fig. 1A, HorA showed ATPase activity in both PE and PC proteoliposomes (respectively 95 nM·mg\(^{-1}\) min\(^{-1}\) and 225 nM·mg\(^{-1}\) min\(^{-1}\)). The higher enzymatic activity in the PC proteoliposomes was reproducible (standard error: respectively, 8 and 30 nM·mg\(^{-1}\) min\(^{-1}\)). This finding contrasts with the results of a simple transport assay using Hoechst 33342, a prototypical MDR substrate that fluoresces upon integration into the lipid bilayer and

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FIGURE 1. Effect of DOPE on HorA activity. A, ATPase activity. HorA proteoliposomes containing either DOPE or DOPC were incubated with 2 mM ATP. The specific ATPase activity was calculated by measuring the release of inorganic phosphate (see “Experimental Procedures”). The data are means of four independent experiments, with error bars representing S.D. B, Hoechst 33342 transport. PE and PC proteoliposomes (about 15 µg of protein) were diluted to 950 µg of reconstituted HorA based on Fourier self-deconvolution (supplemental Fig. S1). After 3 min, Hoechst 33342 was added to the suspension. Once the Hoechst fluorescence had reached a steady level, ATP was added (arrow) to a final concentration of 2 mM. The fluorescence intensity before addition of ATP was normalized to 100%.

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[Diagram A: ATPase activity graph showing DOPE and DOPC.]
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Conformational Modifications Caused by Lipid Change—Two biophysical approaches, FTIR and EPR, were then used to probe whether structural changes were associated with the functional differences observed between the two lipid environments.

Secondary Structure Changes—ATR-FTIR probes global changes in protein conformation. The analysis was performed on the amide I region of deuterated samples in order to distinguish the α-helical secondary structure from the random coil (13). PE and PC proteoliposomes showed a typical spectrum for reconstituted protein, with indistinguishable amide I bands, indicating that HorA adopts identical secondary structure in the two environments (Fig. 2). Fourier self-deconvolution and curve-fitting of these spectra identified a 62% β-sheet content, a 19% β-turn content, and 10% random structures, in good agreement with the crystal structures of Sav1866 (18) and MsbA (19).

Orientation of HorA Transmembrane Domains—When deposited on a germanium plate, lipid membranes tend to align parallel to the reflection element (with their acyl chains perpendicular to it). This makes it possible to measure the average orientation of reconstituted proteins using polarized light (11). IR spectra were recorded with incident light light polarized parallel and perpendicular to the germanium plate. Dichroism spectra were computed by subtracting the parallel- from the perpendicular-polarized spectrum (Fig. 3A). In this set-up, a positive deviation of the dichroism spectrum indicates a dipole oriented preferentially perpendicular to the membrane, and a negative deviation of the dichroism spectrum indicates a dipole oriented along the membrane plane (11). The dichroism spectra for both proteoliposomes displayed a positive signal in the area of amide I, indicating an orientation of the backbone C=O vectors mainly perpendicular to the germanium plate. The maximum of this peak was located at 1658 cm−1 (Fig. 3B) for both types of proteoliposomes, indicating that most of the dichroism arises from α-helical structures. These two dichroic spectra thus correspond to α-helices oriented roughly perpendicular to the germanium plate.

By calculating the dichroic ratio it is possible to obtain more quantitative information about molecular orientations from the IR spectra (20). The dichroic ratio R_{ATR} is defined as the ratio of the amide I peaks of the parallel-polarized and perpendicularly polarized spectra (see “Experimental Procedures”). The calculation can be limited to the helical regions by computing the R_{α-hel} solely from the α-helical components of the amide I peaks. For HorA, this signal should come mostly from the TM helices as, according to the available crystal structures, they represent about 80% of the helical structure content of ABC exporters. Table 1 shows that in PE liposomes, the average angle of the HorA backbone C=O vectors of α-helices was 41° ± 2° with respect to the membrane plane normal. This is in good agreement with the average angle calculated for the carbonyl vectors involved in α-helices in the nucleotide-free structures of both P-gp (21) and MsbA (19). In PC proteoliposomes, the average α-helical backbone carbonyl angle appeared signif-
Significantly reduced, by about 10° (absolute value: 31°/H11006 2°). This indicates an overall tightening of the TMD bundle in the PC environment.

Kinetics of HorA Deuteration—The solvent accessibility and stability of a protein can be assessed by measuring hydrogen/deuterium exchange at constant pH and temperature (23, 24). H/D exchange in HorA was studied by monitoring the amide II (δ(N-H) absorption peak decrease as a function of time (see “Experimental Procedures”) upon exposure to a D2O-saturated N2 flow (Fig. 4, A and B).

The H/D exchange rate was quantified by monitoring the evolution of the amide II peak area for PE and PC proteoliposomes between 0 and 100% (see “Experimental Procedures”). Remarkably, HorA appeared protected from H/D exchange in PC proteoliposomes, with only about 45% exchange after 2 h, as compared with 65% exchange in PE proteoliposomes (Fig. 4 C).

This further highlights structural differences in the protein between the two environments. It also implies that about 140 residues, in each monomer, are involved in this structural change.

Electron Paramagnetic Resonance—HorA possesses a single endogenous cysteine at position 180, located in the middle of the fourth transmembrane helix and corresponding to Thr-177 in the sequence of Sav1866 (Fig. 5A). We used this natural Cys to label HorA with MTSSL spin label and to compare EPR parameters in the two different lipid bilayers. Fig. 5B shows typical EPR spectra for both environments: the spin label in the PC proteoliposomes (dotted line) shows an increase in the mobile component suggesting changes in the local environment of the spin label (solid line).

We also determined spin label accessibility to molecular oxygen or NiEDDA, which partition, preferentially, in a hydrophobic or hydrophilic environment (Fig. 5C) respectively. Although accessibility to NiEDDA appeared equivalent in both proteoliposomes, significantly higher spin label accessibility to molecular oxygen was observed in PC proteoliposomes suggesting that Cys-180 is more exposed to the hydrocarbon phase in the PC environment. Higher exposure to the lipid phase would also explain the higher mobility of MTSSL in PC than in PE proteoliposomes, where the probe should be conformationally restricted by neighboring side chains.

**DISCUSSION**

Phosphatidylethanolamine is the major lipid in bacterial membranes, so it is an obvious choice for testing how lipid-protein interactions affect the function and structure of bacterial membrane proteins. The importance of PE in protein function has been evidenced in a number of cases. Dowhan and co-workers (7, 25, 26) have proposed that it acts as a molecular chaperone for the lactose permease (LacY) of *E. coli*, as its depletion leads to an aberrant conformation of the protein and loss of activity. Replacing PE with PC leads to normal protein topology, but without activity (27). In the case of the secondary multidrug transporter of *L. lactis* LmrP (structurally related to LacY), we have shown that the loss of activity observed when PE

**TABLE 1**

| Lipid   | R\textsuperscript{iso} | R\textsuperscript{\text{-hel}} | Mean angle |
|---------|------------------------|--------------------------|------------|
| DOPE    | 1.65 ± 0.03           | 3.0 ± 0.3               | 41 ± 2°    |
| DOPC    | 1.45 ± 0.02           | 4.0 ± 0.4               | 31 ± 2°    |

**FIGURE 4.** Kinetic of H/D exchange. A, spectra of PE and PC proteoliposomes were recorded as a function of time of exposure to a D2O-saturated N2 flux. B, deuteration leads to a decrease of the amide II band intensity. C, proportion of unexchanged amide bonds in HorA was monitored during deuteration in both PE (○) and PC proteoliposomes (●). It was calculated from the area of the amide II band during deuteration. The data are means of three independent experiments, with error bars representing S.D.
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Lipid Composition Affects HorA Structure—Structural changes in HorA between the two environments were identified by four different approaches based on two independent techniques. The EPR spectra of MTSSL-labeled HorA show a mobile component in PC proteoliposomes that is reduced in PE proteoliposomes. This indicates that position 180, in the fourth transmembrane helix, is differentially constrained in the two types of liposomes. As DOPE and DOPC were the main lipids used, the acyl chains are strictly identical in both conditions. This suggests that the mobility change must be due to a difference in the conformation or orientation of TM4. This is confirmed by the greater accessibility of the spin label to molecular oxygen in PC liposomes than in PE liposomes. In parallel, our ATR-FTIR data also clearly reveal structural differences. In particular, the difference in dichroic ratio indicates a decrease in the average angle (by about 10°) of the carbonyl groups implicated in α-helices in PC proteoliposomes, which can be interpreted as a closing of the TM bundle. Conformational plasticity is expected at the level the α-helices, as crystallographic and spin label studies have shown that ABC transporters can undergo major conformational changes during their catalytic cycles. The “open” state of MsbA shows a wide opening between the two monomers, with the NBDs separated by about 50 Å; once the nucleotide is bound, these domains come in close contact (less than 6 Å) (19). It is remarkable, however, that in the case of HorA, the lipid environment appears to directly modulate this conformational equilibrium, with phosphatidylcholine inducing closure of the gap between helices. A tighter helical bundle could also explain why HorA appears partly protected from H/D exchange in PC liposomes, as closing of the structure should bring the NBDs together, possibly decreasing the solvent exposure of the cytoplasmic domain.

Crystal structures demonstrate that the nucleotide-binding pocket is formed when the two NBDs come in contact, and not when they are apart in the open conformation. Hence, a closing of the TM bundle is likely to favor ATP binding by decreasing the energy barrier of binding pocket formation. This could explain why the ATPase activity of HorA is twice as high in the PC environment as in the PE environment. Interestingly, ABC transporters often show a higher ATPase activity in detergent then in proteoliposomes (18), indicating that embedding the TM domain in a lipid bilayer affects the rate of hydrolysis. The lipid might structurally constrain the α-helices so as to prevent excessive basal activity. Likewise, the increase in HorA ATPase

spectra of MTSSL-labeled HorA reconstituted in PE and PC proteoliposomes. Replacement of DOPC with DOPE induces the appearance of a mobile component (marked) in the EPR spectral line shape. D accessibility of the MTSSL probe was assessed by quenching with molecular oxygen (filled bars) or NIEDDA (empty bars). Data are means of three independent experiments, with error bars representing S.D.
activity observed in PC might be interpreted as resulting from lesser structural constraints imposed by the lipid on the TM domain. In support of this view, EPR shows an increased mobility of Cys-180 in the PC environment (Fig. 5).

ATPase and Transport Activities of HorA Are Uncoupled in PC Proteoliposomes—Our transport assay shows that HorA loses its ability to transport a substrate (or at least Hoechst 33342) when PE is replaced with PC. Although a gross deformation of the protein could cause such an effect, the fact that ATPase activity is retained (actually doubled in PC versus PE proteoliposomes), along with our FTIR data demonstrating an identical secondary structure in PE and PC proteoliposomes, show clearly that the lipid substitution does not induce such a change.

There are mainly two ways to explain the loss of transport with maintained ATPase activity, and these two explanations are not mutually exclusive. On the one hand, since the structure of the transmembrane domain is modified in PC liposomes, the binding pocket of the ligand might be perturbed in this environment so as to prevent or hinder binding. Measuring the affinities of lipophilic ligands for membrane proteins is particularly difficult, as the labeled ligand partitions mostly into the lipid phase, producing a strong background signal. One strategy relies on the use of cross-linkable ligand analogs to allow separation of the labeled protein from the membrane by electrophoresis. Even so, the nonspecific signal may remain very strong, as indicated by experiments in which we compared the apparent affinities of HorA-ethidium bromide binding in PE and PC proteoliposomes, using a cross-linkable monoazide ethidium bromide. Identical labeling was found in both cases (data not shown), but control experiments highlighted the difficulty of reducing the nonspecific signal (mostly due to the long-lived free azide radical) to a level allowing reliable interpretation of the data. In the absence of conclusive binding data, we cannot rule in or out that the conformational modification observed in PE leads to a significant structural modification of the binding pocket, preventing Hoechst 33342 binding and transport.

Alternatively or perhaps additionally, replacing PE with PC might hinder transport by altering propagation of the structural change from the NBDs to the transmembrane domain. Crystal structures of ABC exporters have been obtained in the absence of substrate (18, 19, 21), but it is assumed that the different conformational states visited during the transport cycle are, to some extent, fairly represented by the various structures obtained for MsbA, Sav1866, and P-gp. These conformations range from the wide open apo state to the closed- apo state and finally to the tightly closed nucleotide-bound state. The structures reveal that the conformational changes can be described as rigid body motions, with combinations of rotations and twisting of the helices (19). Throughout the cycle, the NBDs remain tightly bound to the $\alpha$-helices via the intracellular helical loops 1 and 2.

In multidrug resistance transporters, this coordinated motion of the transmembrane and cytoplasmic domains is expected to induce reorganization of the putative ligand-binding pocket, which would shift from a membrane-exposed high-affinity conformation to a solvent-exposed low-affinity state, leading to substrate release into the extracellular medium. Changing the lipid composition might conceivably alter such fine-tuned motion by affecting the local conformation of the transmembrane region. This could lead to transport loss even if some binding affinity was preserved.

Conformational Regulation by Phosphatidylethanolamine—How does replacement of PE with PC lead to a conformational change in HorA? We see two possible explanations: a change in the bulk physico-chemical properties or a change in specific lipid-protein interactions. Several physico-chemical properties of PE and PC bilayers are different, such as phase transition temperature or intrinsic curvature. As in our experiments PE or PC constitutes about 70% of the liposome composition, the bulk properties of the proteoliposomes might be expected to differ. A number of properties, such as viscosity or lateral pressure, are typically described as depending mostly on the acyl chains themselves. As we have used here lipids with identical acyl chains (DOPE and DOPC), it seems unlikely that such properties are involved in the structural changes discussed here. In contrast, properties such as intrinsic curvature depend on the headgroup, because they roughly reflect the balance between the hydrophobic forces of the acyl core and hydration of the headgroup. The activity of certain membrane proteins is proposed to be significantly influenced by modification of such properties of the bilayer, which would directly influence the structure of these proteins. Clearly, this could be the case here, although curvature can probably be ruled out considering that both types of proteoliposomes show similar sizes. Future experiments using various sets of lipids with different physico-chemical features should help decipher how bulk properties affect HorA structure.

Alternatively, HorA might interact specifically with the headgroup(s) of one or several PE molecules. This interaction might be necessary (i) to enable HorA to adopt its native structure or (ii) to allow required conformational changes in HorA during its catalytic cycle. PE is a much stronger hydrogen donor than PC, and several crystal structures have shown direct interactions between negatively charged side chains and the PE headgroup (28, 29). In the case of secondary transporters, we have proposed that a highly conserved Asp in the MFS family may be required for proton gradient-dependent conformational changes (8). This functionally important Asp may in fact belong to a larger conserved PE-binding motif, where a combination of positively and negatively charged side chains would allow bidentate binding to the phosphate and ethanolamine moieties of PE (22).

Analysis of the structures of MsbA, Sav1866, and P-gp reveals that several negatively charged side chains reside close to the supposed locations of the lipid headgroups. Identification of a conserved PE-binding motif in ABC transporters is currently hindered by a lack of data on the lipid dependence of this protein family. In addition, such a motif might involve side chains that are spatially close but distant in the sequence, which would prevent detection by sequence-based approaches. If identified, such a motif could easily be tested by site-directed mutagenesis: its alteration could be expected to cause transport loss with maintained ATPase activity.
To our knowledge, HorA is the first ABC transporter to show a structural dependence on lipid composition, and this study is the first to show that lipid composition can modulate both the activity of the protein and the structure of its native state. Further analysis is required to decipher the molecular mechanisms underlying the PE-dependence of HorA and to test whether other ABC transporters are functionally and structurally influenced by the composition of their lipid environment.

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