Effects of lipid environment on the conformational changes of an ABC importer

Austin J Rice¹, Frances JD Alvarez², Amy L Davidson² and Heather W Pinkett*¹

¹Department of Molecular Biosciences; Northwestern University; Evanston, IL USA; ²Department of Chemistry; Purdue University; West Lafayette, IN USA

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Abbreviations: ABC, ATP binding cassette; CW-EPR, continuous wave-electron paramagnetic resonance; MTSL, S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrrol-3-yl) methylmethanesulfonothioate; PC, phosphatidylcholine

Introduction

Integral membrane proteins play many essential roles from cellular signaling to the transport of molecules of various sizes and chemotypes.¹ It is important to understand how these membrane-embedded proteins interact with the lipid molecules found in their natural environment.² Transmembrane (TM) proteins insert into the lipid bilayer allowing for the non-polar regions to contact the hydrophobic lipid tails and polar regions to be exposed to the aqueous intra- or extracellular spaces.³ Detergents are often used to work with TM proteins in vitro because they mimic the lipid bilayer and prevent protein unfolding and aggregation.⁴ The detergent micelle can alter the activity and regulation of a protein⁵ and may have effects on conformational shifts.⁹-¹² This necessitates the study of TM proteins in a lipid bilayer.

Analysis of protein dynamics and conformation change has been greatly facilitated by site-directed spin-labeling electron paramagnetic resonance spectroscopy (SDSL-EPR).¹³,¹⁴ An EPR-sensitive spin-label is covalently attached to cysteine residues engineered at specific sites in the protein. Analysis of the EPR spectrum allows us to determine the mobility of the spin-label and the distance between spin-labels. EPR spectroscopy only detects lone-pair electrons such as those provided by the stable free-radical group of spin-label or certain oxidation states of transition metals. Since most lipids, detergents, and aqueous buffers do not contribute to the EPR spectrum, EPR spectroscopy is very advantageous for mechanistic studies in a lipid environment.¹⁵

The differences between a lipid bilayer and a detergent micelle begin with variances at the molecular scale. The detergents used for structural studies often have an uncharged head group that is large relative to the non-polar tails (Fig. 1A). This gives the molecule a cone shape, which forms micelles with a small radius.¹⁶ Commonly used lipids have a variety of head groups and two lipid tails that give the molecule a roughly cylindrical shape that enables formation of lipid bilayers (Fig. 1B). The lipid composition of a bilayer has a well-documented effect on TM protein function,¹⁷,¹⁸ which can be roughly divided into direct and indirect interactions of lipid with protein. “Co-factor” lipid molecules can interact directly with a TM protein at specific sites with relatively high affinity,¹⁹,²⁰ such interactions can be vital for protein function and stability.²¹,²² Other lipid-protein interactions are direct yet non-specific, involving the ring of annular lipids surrounding the TM protein. Often the hydrophobic region of a TM protein will have grooves or cavities where lipids or amphipathic molecules bind and affect the activity of the protein.²³ Bulk properties of lipid bilayers such as thickness, lateral pressure, viscosity, and curvature may affect protein function and stability by altering the energy changes involved in conformation change.²⁴ For example, if the thickness of the annular lipid bilayer does not match the thickness of the TM region, either the protein or lipid will alter its structure to minimize the mismatch. However, altering the bilayer thickness is a high energy solution, so hydrophobic
attached to N93C.31,32 For this study, we also probed a third cytoplasmic gate closes upon nucleotide binding, which could be probed via site-directed CW-EPR at residue N93C. This gate is formed by a loop between TM helices 2 and 3 and effectively opening in the presence of nucleotide. The cytoplasmic gate undergoes subtle conformation shifts, which may control the flux of substrate through the central translocation pathway. The periplasmic gate undergoes subtle conformation shifts, which may control the flux of substrate through the central translocation pathway. This family of membrane proteins includes many potential drug targets, which take up vital nutrients and cofactors and may be found in many pathogenic bacteria, so understanding the mechanism, especially in a lipid environment.7,9,10,28,29 While this gate did not close, and thus was not considered a gate in a small substrate transporter like MolBC, conformational changes at this site provide valuable information on the effects of lipid composition on protein conformation.

To test the effects of lipid composition on the nucleotide triggered movement of sites along the transmembrane domain, we attached nitroxide based spin labels to two positions in the translocation channel, N93C and I151C. MolBC-A was then reconstituted into different lipid extracts and tested for activity and conformational changes at spin-labeled N93C and I151C. EPR analysis of MolBC-A at both sites indicated that lipid restricted the conformational changes along the TMD, though some lipid types restricted movement more than others. Similarly, the type of lipid extract affected the ATP hydrolysis activity of the protein. Our results show that technical factors such as reconstitution efficiency and sample viscosity also varied in the different extracts. Our purpose in this work is to share observations from our studies and methodology, which may be applicable in studies to determine protein dynamics in a lipid environment.

Results

When choosing the lipid into which MolBC would be reconstituted, we had the option of lipids synthesized with a known head group and tail length or lipids extracted from cell membranes with different levels of purity (Fig. 1C). For example, phosphatidylcholine (PC) lipid could be extracted and purified from soybean or chicken egg, in which case PC is the most abundant head group and tail lengths vary in proportions. Alternatively, an extraction of all lipid species from an organism can be collected (e.g., E. coli total lipid extract) and optionally fractionated (E. coli polar lipid extract) eliminating some of the lipid species from the extract. The mixture of lipid chemistries found in the extracts confers some advantages in our mechanistic studies. First, if a TM protein required an unknown co-factor lipid for optimal activity, an extract has a greater chance of containing this potential co-factor. Second, the risk of hydrophobic mismatch is mitigated by the variety of tail lengths present in the extract; MolBC would pair with the appropriate length of lipid to form the lowest energy state. Third and most important, the mixture of lipid chemistries conferred by an extract better resembles the natural environment of the plasma membrane.33,34
Three lipid extracts were chosen for these studies due to their frequency of use in EPR membrane protein studies or their similarity to the native environment for MolBC. The first lipid selected, Soybean PC, is a lipid extract containing 14–23% PC in addition to phospholipids with other head groups such as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The hydrophobic tails contain 1% C14:0, 36% C16:0, 2% C16:1, 20% cyclopentyl-C17:0, 1% C18:0, 28% 18:1, 12% cyclopropyl-C19:0. We mixed this lipid extract with Egg PC (99% PC; Avanti: 840051C, product information) in a 3:1 ratio following the lead of experiments conducted on another ABC transporter. The third lipid selected was E. coli total lipid extract, which did not undergo the additional fractionation of E. coli polar extract. The major head groups are 67% PE, 23% phosphatidylglycerol (PG), 9.8% cardiolipin (CA). The hydrophobic tails contain 1% C14:0, 36% C16:0, 2% C16:1, 20% cyclopentyl-C17:0, 1% C18:0, 28% 18:1, 12% cyclopropyl-C19:0. We mixed this lipid extract with Egg PC (99% PC; Avanti: 100500C, product information). The tail content should be similar to the polar lipid extract described above. The E. coli total and polar extracts are closer to the native distribution lipids found in the organism of origin for MolBC-A, Hemophilus influenzae, which tend to have short tail lengths. For example, 35 tested strains of H. influenzae contained 12.7 ± 2.3% C14:0, 11.6 ± 1.08% 3-hydroxy-C14:0, 40.6 ± 2.41% C16:0, 31.4 ± 2.41% C16:1, 2.4 ± 1.13% C18:0, 0.5 ± 0.16% C18:1, and 0.2 ± 0.15% C18:2.

For high signal to noise in an EPR spectrum, it is important to have a high concentration of spin-labeled protein. The reconstitution of TM protein into a lipid bilayer is a major factor that can limit the final protein concentration and required optimization to improve the quality of the final sample. A lipid to protein ratio of 10:1 (w/w) was chosen to maintain a stable reconstituted sample with enough excess lipid to mitigate the effect of residual detergent. This ratio was kept low relative to standard liposome ratios to maximize the final protein concentration in the sample. Reconstitution efficiencies into the E. coli polar lipid mix (77 ± 5%) and E. coli total lipid (72%) were often greater than the efficiency of Soybean PC (68 ± 7%).

Another factor that limits the final sample protein concentration is viscosity; a liposome sample can be easily concentrated by ultracentrifugation, but for EPR analysis the proteoliposome sample needs to be resuspended and fluid enough for loading and removal from a thin 50μL capillary. We found that the proteoliposome samples made with Soybean PC or E. coli polar lipid: Egg PC mixes were notably less viscous at 23 °C when compared with E. coli total lipid samples when resuspended to a similar volume.

For our studies on ligand-dependent conformation change of MolBC-A in a lipid environment, we were interested in the effect of lipid on protein activity. We found that reconstitution of MolBC from detergent to liposome decreased the basal (substrate-free) levels of ATP hydrolysis 3 to 24-fold (Table 1). Different lipid extracts had different effects on activity, with the two E. coli extracts showing higher specific activity than the Soybean PC proteoliposomes. However, all samples have a moderate capacity to bind and hydrolyze ATP. Therefore, incubation periods were timed so that a large majority of the transporter population should have bound ligand.

Continuous wave-EPR spectroscopy indicates that reconstitution of MolBC from detergent to lipid affects the conformation cycle of the transmembrane domain (TMD), as reported by the spin labels at N93C and I151C. Spin-coupling is a measure of the proximity between spin-labels and can be qualitatively identified by broadening in the low-field region of the EPR spectrum. When the spin-labels assume a closer conformation the broadening shifts further down field; when broadening cannot be observed the spin-labels are beyond the detectable distance for CW-EPR spectroscopy (~1.8nm).

When the TMD was probed at N93C (Fig. 2A), we are able to observe spin-coupling in the EPR spectrum (Fig. 2B). Comparing detergent-solubilized MolBC with lipid reconstituted transporter, we see that the reconstituted sample shows less spin-coupling (Fig. 2B). When nucleotide-free (apo) MolBC_N93C spectra are simulated and quantified, we see that both detergent and lipid give a population of three distances 0.8, 0.95, and 1.2nm, though in lipid the larger distance is weighted more (21% in lipid vs 6% in detergent). This suggests that a wider spaced population of spin-label rotamers is stabilized by the lipid bilayer. In addition, we see a shift from predominately mobile spin-label in detergent to immobile spin-label in lipid, indicating that a lipid bilayer keeps the spin-label in a more restricted conformation. The presence of ATP did not alter the mobility in either sample, but spin-coupling was increased (Fig. 2C). When quantified, the majority of the spin for both detergent and lipid shifted to a close distance (at about 0.6nm), indicating a closure of the cytoplasmic gate. Residual spin at 0.9nm in the nucleotide-bound liposome sample indicates that the conformation shift in liposomes was not as complete as it had been in detergent. Addition of magnesium chloride allowed MolBC to hydrolyze the ATP and revert back to the apo state.

| Mutant   | Lipid              | Specific activity (nmol Pi/mg enzyme/min) | Standard error of Activity (nmol Pi/mg enzyme/min) |
|----------|--------------------|------------------------------------------|--------------------------------------------------|
| N93C+MTSL| DM detergent       | 1790                                     | 66                                               |
| N93C+MTSL| Soybean PC         | 200                                      | 16                                               |
| N93C+MTSL| E. coli polar: Egg  | 415                                      | 6                                                |
| N93C+MTSL| PC (3:1)            | 526                                      | 8                                                |
| I151C+MTSL| DM detergent      | 1782                                     | 87                                               |
| I151C+MTSL| Soybean PC         | 73                                       | 5                                                |
| I151C+MTSL| E. coli polar: Egg  | 288                                      | 7                                                |
|          | PC (3:1)           |                                          |                                                  |

Table 1. ATP hydrolysis activity for MolBC in liposomes
to an apo-like conformation with the spin-labels at N93C slightly separated (Fig. 2D).

For site I151C, we also see a decreased mobility of spin-label when reconstitution from detergent to lipid (Fig. 2E). The change in mobility suggests that the lipid dependent conformation shift affects multiple residues along the translocation pathway. However, unlike N93C, liposome reconstitution does not affect the general conformation cycle at I151C, at least not with a mix of E. coli polar lipid and Egg PC. In both detergent and lipid, I151C assumes a close but not closed conformation apo and post-hydrolysis (Figs. 2E and 2G). And with ATP-bound, no broadening is observed, suggesting a widely separated conformation at I151C (Fig. 2F). The reconstitution of MolBC from a detergent micelle to a lipid bilayer appears to decrease spin mobility at both sites tested along the TMD and weaken conformation shifts at N93C.

Continuing our EPR studies in liposomes of different lipid content, we see that the type of lipid does effect conformation change at both TMD sites. In the apo state, soybean PC and E. coli polar lipid mix appear to hold N93C in similar conformations (Fig. 3A). However, the nucleotide-dependent closure of the cytoplasmic gate at N93C is not observed in Soybean PC, while it is clearly observed in E. coli polar lipid mix. Alternatively, E. coli total lipid extract and E. coli polar lipid extract mix appear to give identical conformation shifts at N93C (Fig. 3B). At I151C, soybean PC does not appear to stabilize the close conformation of spin-label (Fig. 3C). A separated state is indicated by the greater intensity at the central peak and less broadening in the low-field spin. As stated above, in E. coli polar lipid mix, spin-label at I151C separates in the presence of ATP, but as there was no observable coupling in the apo state of soybean PC samples, we cannot confirm or rule out conformation change at I151C in soybean PC. Post-hydrolysis, we see again that soybean PC does not support a close conformation, though the difference between the two lipid types is less noticeable, which may be due to inefficient ATP hydrolysis in the reconstituted sample. As in the E. coli lipid mix, spin-label at I151C is mostly immobile in soybean PC, though there is a significant fraction of highly mobile spin indicative of denatured protein. This indicates that MolBC is less stable in soybean PC than the E. coli lipid extracts.

**Discussion**

It is immediately clear that detergent and lipid provide different environments for MolBC. Reconstitution into proteoliposomes is known to eliminate basal levels of ATP hydrolysis for the Type I importer MalFGK assayed in the absence of substrate binding protein. And the basal activity of the Type II transporter BtuCD decreases from
980 to 200nmol Pi/mg enzyme/min upon reconstitution from LDAO detergent to liposomes. We observed a similar reduction for MolBC when reconstituting from DM to liposomes (Table 1). The most likely explanation for this effect on activity would be that a lipid environment slows or otherwise alters the conformation cycle required for ATP binding, hydrolysis, and release.

The difference between the detergent and lipid environments is also confirmed by CW-EPR spectroscopy. At the TMD cytoplasmic gate (N93C), a detergent micelle allows for a greater percentage of transporters with closed gates in the presence of ATP. Moreover, at both TMD sites and independent of nucleotide, the spin-labels shift from high mobility in detergent to low mobility in lipid. These data indicate that there are more rotameric sub-states available to spin-label when MolBC is embedded in a detergent micelle vs. a lipid bilayer. Possible explanations for this lipid effect can be found in the literature. Molecular crowding and lateral pressure applied by the lipid bilayer may be the source of this lipid-effect. Simulation studies with the α-helical protein GlpE and the β-barrel OmpA have indicated that a detergent micelle allows slightly greater flexibility to TM secondary structure relative to a simulated lipid bilayer. Similarly, the LCP structure of R. sphaeroides photosynthetic reaction center indicated a slight compression of TM helices relative to the indetergent structure. Figure 4 illustrates the potential differences between the detergent and lipid environments, with MolBC surrounded by a thin detergent micelle (Fig. 4A; which may be better described as “a prolate ring” of detergent) or a lipid bilayer (Fig. 4B). The detergent ring allows the protein to properly fold and undergo conformation change, but we can expect that it will enforce different restrictions on ATP-driven conformation change. Moreover, the lipid bilayer incurs a significant energetic cost upon deformation, which would resist conformation change in MolBC.

The results we observed in different lipid extracts were more unexpected, indicating the significance of lipid composition. The E. coli lipid extracts allow similar levels of moderate ATP hydrolysis and conformation change at N93C. However, the Soybean PC extract diminished ATP hydrolysis to a greater extent and did not allow significant nucleotide-dependent conformation change at both TMD sites. It is reasonable to assume that transport-associated conformation change will be observed in more native-like lipids. Looking for a specific cause of the soybean PC effects, we can rule out the relatively high concentration of phoscholine head groups, since the E. coli polar: Egg PC mix had a similar percentage (25%). It is more likely that the shorter lipids present in the E. coli extracts supported the proper conformation changes of MolBC, accounting for the higher activity and greater conformational flexibility in these extracts relative to SoyPC. The heterogeneous nature of extracts limits our ability to pinpoint a particular cause of the different lipid effect. However, our findings highlight the importance of testing multiple lipid compositions when analyzing protein conformation change.

This study on MolBC-A highlights the importance of comparative analysis of EPR data in multiple environments. Combined, the results suggest that a lipid bilayer forms a more restrictive environment for the MolBC transporter relative to detergent micelles, where spin-label rotamer shifts and ligand-dependent conformation shifts are more limited. Moreover, we found that some lipid extracts were more restrictive than others. The more native-like mix of E. coli polar lipid and Egg PC offered our MolBC studies a good balance of reconstitution efficiency, sample viscosity, protein activity, and conformational freedom.

**Materials and Methods**

**Mutagenesis, protein purification and spin-labeling** We have previously described the initial cloning, overexpression, mutagenesis, and purification of MolBC-A. Briefly describing our methods of purification and spin-labeling, cells with overexpressed MolBC_N93C were lysed in the presence of n-decyl-β-D-maltopyranoside (DM; Anatrace: D322) detergent and centrifuged. Lysate was loaded onto a Ni-NTA affinity column and washed. MTSL was added to a final concentration of 0.1mM and incubated with mixing at 4 °C for ~16 hours. Free-spin was removed via 80 column volumes of washing. Spin-labeled MolBC_N93C was eluted in 250mM imidazole, then dialyzed and concentrated to at least 120µM. A 27µL aliquot of concentrated, detergent-solublized, spin-labeled MolBC_N93C was used to quantify spin-labeling efficiency via CW-EPR.

**Lipid preparation** Chloroform-dissolved E. coli Polar lipid (Avanti: 100600C) and Egg PC (Avanti: 840051C) were mixed in a 3:1 (w:w) ratio. The chloroform solvent dissolves both lipids and facilitates the formation of liposomes with uniform lipid content. A known
amount of either *E. coli* Polar lipid: Egg PC lipid mixture or *E. coli* total lipid (Avanti: 100500C) was aliquoted into glass vials with Teflon coated caps. Lipids were dried under a stream of Argon while vortexing. Vortexing ensured that a thin layer of lipids was deposited on the walls of the vial. Residual chloroform was removed under vacuum for 16hrs and dry lipids stored at -20 °C for subsequent use. Soybean PC lipid extract (Sigma: P5638) was shipped dry and was resuspended directly in aqueous buffer. The lipids were resuspended to 50mg/mL in 25mM Tris; pH 7.5; 100mM NaCl. To mitigate lipid oxidation, it was preferred to use degassed buffers and work under argon as much as possible. But working with the above lipid extracts, degassing did not affect the EPR results. A cloudy lipid suspension was produced by vortexing. The sample was sonicated 10 s on/ 10 s off for a total on time of 5min using a micro-tip probe sonicator (amplitude = 10–40 for samples ranging from 0.2 to 2mL respectively). Glass vials containing the lipid solution were clamped and incubated in room temperature water to disperse heat during sonication. A decrease in the optical density of the solution indicated that sonication successfully broke up the multi-laminar lipid sheets of the suspension and formed liposomes. Liposomes were kept covered, on ice, and used within a few hours of sonication.

**Biobead preparation and use**

Approximately 2mL biobeads (Bio-Rad: 152–8920) were washed with 2x10mL methanol, 3x10mL water, then 3x10mL 25mM Tris; pH 7.5; 100mM NaCl. To make the biobeads more effective at absorbing detergent, they were allowed to soak in buffer overnight before use. In order to measure the volume of dried biobeads, a clipped and calibrated Eppendorf pipette tip (P100 or larger) was used. Clipping the tip made the aperture large enough for biobeads to enter; the beads were allowed to settle in the tip forming a plug that reached the calibration mark. Excess liquid was then dispensed, leaving a plug of dry biobeads. Biobeads were then transferred to lipid protein solution as described below.

**Protein reconstitution**

The liposomes were dissolved with 40-80mM Sodium cholate. Different components of lipid extracts required more cholate than others to dissolve. An optical density measurement equal to buffer verified that 40mM cholate was the minimum concentration required to completely dissolve Soybean PC and *E. coli* Total extract liposomes (*E. coli* polar: Egg PC (3:1) liposomes required 80mM cholate ). Dissolved lipid and purified protein were mixed in a 10:1 (w:w) ratio, incubated on ice for 5min, then biobeads were added to the sample (2/3 of the sample volume in dry bio beads). Samples were incubated at 4 °C for -16hrs with rocking, then at room temperature for 2–3hrs. The biobeads slowly removed the cholate (and residual DM from the protein purification), forming proteoliposomes. Samples were diluted with 25mM Tris; pH 7.5; 100mM NaCl to fill a Ti 45 rotor centrifuge tube (Beckman: 355622 -73mL) and centrifuged at 150,000xg for 2hrs at 4 °C. The dilution and centrifugation steps aided liposome formation by diluting detergent and removing residual detergent-stabilized protein. Proteoliposomes were resuspended in a minimal volume of 25mM Tris; pH 7.5; 100mM NaCl. Reconstitution efficiency was calculated by determining the spin/protein ratio in the detergent-solubilized sample with the protein concentration determined via BCA protein assay (Thermo Scientific: 23250); the spin/protein ratio was used to calculate the amount of protein in a liposome sample, which was divided by the amount of protein reconstituted.

**ATP hydrolysis Assay**

An enzyme linked inorganic phosphate assay (Cytoskeleton Inc.) was used to assay rates of ATP hydrolysis. Assays were set-up in a UV transparent 96-well, half-area plate with 0.2mM 2-amino-6-mercapto-7-methylpurine riboside, 0.1 unit purine nucleoside phosphorylase, 1mM MgCl₂, and MolBC. Each assay was 100µL, buffered with 25mM Tris pH 7.5; 100mM NaCl. 10–30µg/mL of MolBC (depending on activity of the mutant) was assayed in triplicate. The assay was started by addition of 2mM ATP. The initial rates of A₃₆₀ increase for replicate samples were compared with determine standard error of the activity. The length of the initial region was varied to minimize standard error, while maintaining a high (> 0.98) r² factor in the linear regression. A standard phosphate curve was used to quantify activity from the assay results. For specific activity calculations, the protein concentration was determined from the CW-EPR data by comparison of the spin concentration (from integrated absorbance peak compared with a 100µM MTSL standard) to the spin-labeling efficiency.

**Continuous wave- electron paramagnetic resonance**

The CW-EPR technique was described previously. Briefly, 25µL sample was aspirated into a 50µL glass capillary and nestled in a quartz capillary. The following ligands were used: MolA at 2x molar excess over MolBC; 10mM ATP + 1.5mM EDTA; and 16.5mM MgCl₂. Ligands were serially added to recovered protein samples, then incubated for at least 10min (60 min for MolA) before being re-scanned. X-band EPR spectra were collected at room temperature (296K) with a Bruker EMX-plus spectrometer with an ER 4119HS cavity, signal averaged 20 times in liposomes and 9 times in detergent. The scan width was set to 300Gauss and clipped to the values described in the figure legends. Distances were calculated using the Short Distances simulation software written by C. Altenbach.

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