Translocation of Phospholipids Is Facilitated by a Subset of Membrane-spanning Proteins of the Bacterial Cytoplasmic Membrane*

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The mechanism by which phospholipids are transported across biogenic membranes, such as the bacterial cytoplasmic membrane, is unknown. We hypothesized that this process is mediated by the presence of the membrane-spanning segments of inner membrane proteins, rather than by dedicated flippases. In support of the hypothesis, it was demonstrated that transmembrane α-helical peptides, mimicking the membrane-spanning segments, mediate flop of 2-[6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproyl (C6-NBD)-phospholipids (Kol, M. A., de Kroon, A. I., Rijkers, D. T., Killian, J. A., and de Kruijff, B. (2001) Biochemistry 40, 10500–10506). Here the dithionite reduction assay was used to measure transbilayer equilibration of C6-NBD-phospholipid(s) in proteoliposomes, composed of Escherichia coli phospholipids and a subset of bacterial membrane proteins. It is shown that two well characterized integral proteins of the bacterial cytoplasmic membrane, leader peptidase and the potassium channel KcsA, induce phospholipid translocation, most likely by their transmembrane domains. In contrast, the ATP-binding cassette transporter from the E. coli inner membrane MsbA, a putative lipid flippase, did not mediate phospholipid translocation, irrespective of the presence of ATP. OmpT, an outer membrane protein from E. coli, did not facilitate flop either, demonstrating specificity of protein-mediated phospholipid translocation. The results are discussed in the light of phospholipid transport across the E. coli inner membrane.

Biological membranes are composed of proteins and lipids, the latter organized as a bilayer. Cellular growth requires influx of new membrane components into the membranes. Because the synthesis of phospholipids, the major lipid constituents of most biological membranes, is generally confined to one leaflet of biogenic membranes (i.e. membranes containing phospholipid biosynthetic enzymes), transport of phospholipids to the other membrane leaflet is required. It has been shown that biogenic membranes, such as bacterial plasma membranes (1–3) and the endoplasmic reticulum membrane (4–7), exhibit rapid phospholipid flip-flop with similar characteristics, including limited sensitivity toward proteolysis, bidirectionality, energy independence, and phospholipid head group independence (recently reviewed in Ref. 8). In contrast to flip-flop in biogenic membranes, phospholipid translocation in model membranes composed of only lipids is very slow (9). It is therefore generally accepted that phospholipid translocation is a protein-mediated process. In some membranes, this activity has been attributed to dedicated proteins (see e.g. Ref. 10); however, the identities of the putative phospholipid translocators, or flippases, in the bacterial cytoplasmic membrane and in the endoplasmic reticulum remain obscure despite many efforts.

Based on the general characteristics of flip-flop, we hypothesized that the mere presence of α-helical stretches of transmembrane proteins is sufficient for flop to occur, rendering the elusive flippases redundant. We showed previously that the presence of synthetic transmembrane peptides, mimicking the α-helical stretches of transmembrane proteins, induces flop of C6-NBD-PL analogues in model membranes (11, 12), supporting this hypothesis.

Bacterial membrane proteins display a large diversity in structure and organization, more than can be accounted for by α-helical model peptides. α-Helical membrane proteins often span the bilayer with several TMHs, whereas the model peptides are single-spanning. Additionally, membrane proteins usually have domains outside the membrane, and in some cases form oligomers. Here we report on phospholipid translocation, induced by a subset of well characterized membrane proteins with different membrane organizations, briefly described below.

Leader peptidase (Lep) from Escherichia coli has two membrane-spanning α-helices and adopts an overall Nout/Cout topology in the inner membrane (IM) of E. coli (13). The large C-terminal catalytic domain is in close contact with the periplasmic leaflet of the IM, interacting with phospholipids (14, 15). Lep is an essential protein (16) involved in membrane biogenesis, as it clips off the signal peptide of proteins that are translocated via the E. coli Sec machinery. Moreover, its purification and functional reconstitution in proteoliposomes have been characterized (17, 18). Taken together, this renders Lep an excellent model protein to test our hypothesis.

The potassium channel KcsA is another well characterized protein of the bacterial cytoplasmic membrane. Its crystal structure has been determined (19). Unlike Lep, KcsA is an oligomeric protein forming a stable homotetramer (20). Each monomer contains two membrane-spanning domains and has an overall Nii/Cin topology. The interaction of KcsA with lipids

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1 The abbreviations used are: C6-NBD-PL, 1-palmitoyl-2,6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproyl-sn-glycero-3-phospho-lipid; DDM, n-dodecyl-β-D-maltoside; IM, inner membrane; Lep, Leader peptidease from E. coli; LPS, lipopolysaccharide; LUVETs, large unilamellar vesicles prepared by extrusion technique; OG, n-octyl-β-D-glucopyranoside; PG, phosphatidylglycerol; –P, inorganic phosphate; PL, phospholipid(s); TLE, total phospholipid extract of E. coli; TMH, transmembrane helix.
has been characterized, as well as the role lipids play in its membrane assembly (21, 22).

The E. coli inner membrane protein MsBA was chosen as a representative of the large superfamily of ATP-binding cassette transporter proteins, or traffic ATPases. In bacteria, the ATP-binding cassette transporters have a complex membrane organization with two hydrophobic domains, each of them typically spanning the membrane six times (23). MsBA is a homodimer, the 64-kDa monomer spanning the membrane with six TMHs (24, 25). It was shown to be an ATPase (26). The MsBA gene was first discovered as a multicopy suppressor of mutations in htrB (24), which encodes a protein involved in the synthesis of lipopolysaccharide (LPS). Overexpression of mshBA strain, LPS precursors and phospholipids accumulate in the inner membrane at the non-permissive temperature (27). Based on these observations and on the recently resolved crystal structure (25), MsBA was suggested to be a (phospholipid flipase, which provided an extra rationale for testing the inner membrane at the non-permissive temperature. MsBA is an ATPase (26). It was shown to be an ATPase (26). The MsBA protein was purified by affinity chromatography on a Ni2+-nitrilotriacetic acid column with 1 M phenylmethylsulfonyl fluoride, 300 ng/ml leupeptin, 10 mM EDTA, pH 7.4, at 20 °C. Lep was prepared from Escherichia coli strain BL21(DE3) carrying a pT7-KcsA plasmid, essentially as described previously (20, 32). Cells were grown for 2 h after addition of isopropyl-1-thio-glucopyranoside and harvested. The membrane fraction was isolated as described above. The membrane pellet was solubilized in 10 mM HEPES, 100 mM NaCl, 5 mM KCl, 10 mM imidazole, and 1 mM dodecylmaltoside (DDM) and applied to a Ni2+-nitrilotriacetic acid column. After washing the column with ~5 volumes of 10 mM and ~5 volumes of 50 mM imidazole in the above buffer, respectively, the protein was eluted with buffer containing 300 mM imidazole and stored at a concentration of 0.64 mg/ml at 4 °C.

His-tagged MsBA was a kind gift from Drs. William Decker and Christian Raetz (28) and was stored at a concentration of ~0.4 mg/ml in 0.1% (w/v) DDM, 200 mM imidazole, 50 mM HEPES, 500 mM NaCl, 5 mM MgCl2, 10% (w/v) glycero, and 5 mM b-mercaptoethanol at 20 °C. OmpF211K/R218E, an OmpT mutant with reduced autotranslocatory activity, was purified and purified as described (33), was generously supplied by Gerard-Jan de Roon and Dr. Maarten Egmond and stored as a stock of 2.6 mg/ml in 10 mM Tris/HCl, 1% octyl-oligo-oxoethylene, pH 8.3, at 20 °C.

Preparation of Large Unilamellar Vesicles by Extrusion (LUVs)—
Vesicles with and without the model peptides H1 and WALP23 were prepared as described previously (11), except for omitting K3Fe(CN)6. Briefly, a mixed film was prepared consisting of E. coli lipid extract (TLE), the indicated amount of peptide, and C8NBD-PG at 0.2 mol % of PL-Pi. The lipid film was hydrated with buffer Z (50 mM triethanolamine, 10 mM KCl, 1 mM EDTA, pH 7.5) to a final concentration of 5 mM phospholipid. After repetitive freezing and thawing, and subsequent extrusion through 200-nm membrane filters (Anotop 10, Whatman, Maidstone UK). unilamellar, sealed vesicles symmetrically labeled with C8NBD-PG were obtained.

Preparation of Proteoliposomes—
Reconstitution of Lept into proteoliposomes was performed by solubilizing PL-Pi in buffer Z and incubating overnight at 4 °C under continuous stirring. The resulting symmetrically labeled proteoliposomes were collected by ultracentrifugation at 293,000 g for 90 min at 4 °C and resuspended in a small volume of buffer Z.

The model peptide WALP23 was reconstituted by octyl glucoside dilution following the same procedure, starting from a mixed lipid film containing the peptide at a 1:1000 molar ratio with respect to PL-Pi. To check whether the method of reconstitution influences the properties of the proteoliposomes with respect to phospholipid translocation, a second protocol was also used. LUVs composed of TLE (~5 mol PL-Pi) prepared in buffer Z were solubilized with octyl glucoside (OG) (1% (w/v) final concentration) resulting in an optical clear solution. Lep was added (1:1000 molar ratio with respect to PL-Pi), and the detergent was removed using Bio-Beads SM (Bio-Rad). Briefly, the octyl glucoside solution was incubated for 30 min under gentle rotation at room temperature; ~80 mg/ml Bio-Beads was added, and incubation was continued for 2 h. Next, the solution was added to 80 mg/ml fresh Bio-Beads and again incubated for 2 h under rotation. Subsequently, the solution was incubated overnight at 4 °C, again with fresh Bio-Beads. The vesicles were collected by centrifugation (1 h at 435,000 × g) and resuspended in buffer Z.

KcsA was reconstituted as described (22), based on a published protocol (15). Briefly, LUVs (~5 mM TLE) containing 0.5%
C₆NBD-PG with respect to total PL-Pₐ, prepared in 10 mM HEPES, 100 mM NaCl, 5 mM KCl were solubilized by adding Triton X-100 to a final concentration of 8 mM. The tetrameric protein was added at a molar ratio of 1:1000 or 1:2000 with respect to PL-Pₐ, as indicated, typically in a final volume of 700 μl. Detergent was removed using Bio-Beads as above.

MsbA was reconstituted according to Doerrler et al. (26) with minor modifications. LUVETs (5 mM TLE-Pₐ) were prepared in 50 mM HEPES, 50 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5, and solubilized with 0.2% (w/v) DDM from a 20% (w/v) stock solution in H₂O₂, yielding an optically clear solution. Protein was added to a 1:1000 molar ratio with respect to PL-Pₐ, in a final volume of 350 μl. Following dilution to 1 ml, detergent was removed with Bio-Beads as above.

OmpF was reconstituted as described for OMPLA, another E. coli outer membrane protein (35). LUVETs prepared from TLE (5 mM PL-Pₐ) and containing C₆NBD-PG (0.5% of total PL-Pₐ) in buffer were solubilized with OG added from a 20% (w/v) stock solution in buffer Z to a final concentration of 1% (w/v) and supplemented with OmpF-T at a 1:1000 molar ratio with respect to PL-Pₐ to form mixed micelles, which were subsequently incubated with Bio-Beads to remove detergent as above.

Flip Assay—All procedures were performed as described previously (11). The LUVETs or proteoliposomes, symmetrically labeled with C₆NBD-PG, were incubated with 25 mM sodium dithionite (Na₂S₂O₄) for 5 min to reduce and thereby quench the fluorescent NBD label in the outer membrane leaflet, followed by gel filtration to remove excess dithionite. The resulting asymmetry labeled vesicles were incubated at a concentration of ~3 mM PL-Pₐ at the temperature indicated. At different time points aliquots of vesicles were taken, and the in-out translocation (flip) of NBD-phospholipids was measured by determining the amount of NBD-phospholipid susceptible to reduction by 8 mM sodium dithionite in 3 min at 20°C. Flip Assay—Proteoliposomes (~2 mM PL-Pₐ) were incubated 30 min on ice with 0.1 mol % NBD-PL, added from an ~1 mM stock solution in ethanol, to allow the probe to incorporate in the outer membrane leaflet. The concentration of ethanol in the vesicle suspension never exceeded 0.2% (v/v). Phospholipid out-in translocation (flip) was initiated by shifting the vesicles to 37°C. At different time points, aliquots were assayed for the transmembrane distribution of C₆NBD-PG as described for the flip assay. The pool of C₆NBD-PG protected against reduction by dithionite was taken as the amount of flip.

Fluorescence Measurements and Calculations—Fluorescence measurements were performed as described (11), in buffer Z unless noted otherwise, using an SLM Aminco SPF 500C spectrofluorometer (excitation 460 nm and emission 534 nm). The percentage of NBD-phospholipid with respect to total PL-Pₐ in LUVETs containing 1% (w/v) octyl glucoside, (excitation 280 nm and emission 350 nm). ATPase activity was determined as described (26).

RESULTS

In this study, we investigated the capacity of membrane proteins to induce helix-mediated phospholipid translocation in proteoliposomes, using short chain NBD-labeled phospholipids as probe molecules. Proteoliposomes are generally prepared by removal of detergent from a mixed micelle solution of lipids, protein, and detergent, in contrast to the previously used peptide-containing model membranes that were prepared by extrusion (11, 12). Residual detergent in the proteoliposomes might compromise membrane integrity, in which case the dithionite reduction assay would not work, or might cause artifacts in phospholipid translocation. Therefore, the model peptide WALP23 was incorporated in vesicles by reconstitution from detergent and by extrusion of multilamellar vesicles prepared from a mixed peptide-lipid film. The resulting proteoliposomes with WALP23, with and without WALP23 incorporated, were compared with respect to their permeability to dithionite, and the occurrence of flip of C₆NBD-PG. The peptide/phospholipid ratios before (1:1000 mol/mol) and after vesicle preparation were similar for the two protocols (not shown).

In both procedures C₆NBD-PG was present during vesicle preparation and thus symmetrically labeled with respect to membrane leaflets. Fig. 1 shows that vesicles prepared by the extrusion protocol and prepared by dilution from octyl glucoside have similar C₆NBD-PG pools protected from dithionite reduction (~35 and ~40%, respectively), irrespective of the incorporation of WALP23. The quenching of the C₆NBD-PG in the outer leaflet is completed in ~3 min, as was shown previously (11). These data demonstrate that both reconstitution protocols yield vesicles that are sealed to dithionite and unila- mellar. The slightly smaller protected C₆NBD-PG pool in vesicles prepared by detergent dilution suggests that these vesicles are relatively small.

Subsequently, the vesicles were treated with dithionite to quench the NBD fluorescence in the outer membrane leaflet. After removal of excess dithionite, the vesicles were incubated at 37°C and assayed at different time points for the reappearance of C₆NBD-PG in the outer leaflet. Fig. 1B shows that at 37°C efficient flip occurs in vesicles containing WALP23 prepared by reconstitution from detergent, albeit at a slightly slower rate than in WALP23-containing LUVETs. The apparent first order flip rate constant at 37°C in LUVETs containing WALP23 at a 1:1000 molar ratio was determined according to Equation 4 and found to be ~0.8 h⁻¹, ~4 times higher than the flip rate constant at 25°C (12), indicating that flip rates increase with temperature. The spontaneous translocation of C₆NBD-PG in vesicles without peptide was slightly increased at 37°C (Fig. 1B) as compared with 25°C (12). Together, these data clearly demonstrate that reconstitution from detergent yields vesicles in which the dithionite reduction assay can be used to measure peptide-induced flip and that any residual detergent does not enhance flip.

Next, we investigated whether leader peptidase (Lep), an inner membrane protein of E. coli, reconstituted by dilution from OG could induce flip. Reconstitution of Lep (starting from a 1:1000 protein/lipid molar ratio) results in protein-containing...
vesicles (Fig. 2A, inset). Recovery of phospholipid and protein after reconstitution are 70 and 80%, respectively. Addition of dithionite (Fig. 2A) reveals that the reconstituted vesicles are sealed and unilamellar, with a protected pool of C6NBD-PG of 32 ± 3%, similar to that of vesicles without protein, which have a protected pool of 35 ± 3%. Fig. 2B shows that the presence of Lep (triangles) enhances flop of C6NBD-PG in vesicles composed of an E. coli lipid extract. The flop rate in vesicles containing Lep was 3-fold higher than that in the absence of protein (Fig. 2B, circles). Because flip-flop in E. coli has been shown to be bi-directional (1), we also investigated Lep-induced flip in proteoliposomes. To this end, the proteoliposomes, prepared by detergent removal with Bio-Beads, were incubated on ice with C6NBD-PG, added from a stock solution in ethanol. After incorporation of the probe into the outer leaflet, the proteoliposomes were shifted to 37 °C to allow phospholipid translocation. Fig. 2B (diamonds) shows that flip of C6NBD-PG induced by Lep is comparable with flop (triangles). Flip proceeds at a rate 2.5 times higher than flip in vesicles without protein (not shown). Thus, phospholipid translocation mediated by Lep proceeds in both directions.

To investigate which part of Lep is responsible for inducing flop, the first transmembrane stretch of Lep (designated H1)
was chemically synthesized and incorporated in LUVETs. As was shown for WALP23 in Fig. 1, also the presence of this membrane-spanning α-helical stretch induced very efficient translocation of C₆NBD-PG (Fig. 2B, squares). Previously, we reported that a positively charged model peptide (AcGKKLα₃-KKA-NH₂, KALP23) was particularly efficient in inducing flop of C₆NBD-PG in vesicles composed of E. coli phospholipids (11). To investigate whether introducing positively charged lysine residues in the membrane-water interface would influence the efficiency of flop induced by H₁, a derivative (H₁'), in which the first three amino acids are replaced by the first three amino acids of KALP23 (GKK), was also tested. The N-terminal modification did not have a significant effect on the translocation efficiency, indicating that primarily the transmembrane part causes phospholipid translocation. It has been reported (14, 15) that the water-soluble P2 domain of Lep interacts with membranes. To investigate whether this contributes to Lep-induced phospholipid translocation, the P2 domain was added to LUVETs at a high protein/lipid ratio (1:50 mol/mol), at only one, or at both sides of the model membrane. P2 was added either after formation of the vesicles thus being present at the outer leaflet only, or during and after vesicle formation, in which case P2 molecules have access to both membrane leaflets. Irrespective of the presence of P2 at one or at both membrane leaflets, no significant flop of C₆NBD-PG was observed during 90 min of incubation (data not shown), again indicating that the transmembrane segment(s) of Lep are responsible for flop. In conclusion, phospholipid translocation induced by Lep is bi-directional and likely to be mediated by the transmembrane stretch(es) of this protein.

To investigate whether Lep is unique in its ability to induce phospholipid translocation, we tested another bacterial inner membrane protein, the tetrameric potassium channel KcsA. The quality of the proteoliposomes with and without KcsA was checked as above. After overnight removal of Triton X-100 by Bio-Beads, ~60% of the phospholipids and ~70% of the protein were recovered, and sealed vesicles were obtained, with a protected C₆NBD-PG pool of 34% in the presence (1:1000 tetramer/lipid molar ratio) and 42% in the absence of protein. Upon reconstitution, KcsA retains the stable tetramer configuration (Fig. 3, inset). At a low protein (tetramer/lipid ratio (Fig. 3, triangles, 1:2000), flop of C₆NBD-PG is somewhat enhanced as compared with vesicles without protein (circles). When the protein is incorporated at a tetramer/lipid ratio of 1:1000, the flop rate is 4-fold increased compared with vesicles without protein, comparable with the rate of flop induced by Lep at a 1:1000 monomer/PL-Pi ratio (Fig. 2). These results show that induction of passive translocation of NBD-PL is not a unique property of leader peptidase but that flop is also facilitated by an oligomeric membrane protein. The rates of translocation for the various proteins and peptides are summarized in Table I. Although helix-mediated flop of phospholipids may provide an important route for phospholipid translocation in the E. coli inner membrane, the existence of dedicated phospholipid transporters in the E. coli inner membrane cannot be excluded. Recently, MsbA was proposed as a candidate lipid flippase (25). MsbA is a membrane-spanning E. coli inner membrane protein with six TMH per monomer, which is functional as a homodimer and hydrolyzes ATP at the cytosolic side of the E. coli inner membrane. This is also the site where phospholipids destined for translocation are synthesized. Because ATP can be conveniently added to pre-existing proteoliposomes (i.e. outside the liposomes), we tested the ability of MsbA to induce flip (rather than flop) of C₆NBD-PL. The inset of Fig. 4 represents a Coomassie-stained SDS-PAGE gel loaded with KcsA before (lane 1, 1.3 μg of protein from a stock solution) and after (lane 2, 2.4 μg of protein) reconstitution in proteoliposomes composed of E. coli PL, at a 1:1000 tetramer/PL-Pi molar ratio.

![Figure 3](http://www.jbc.org/Downloaded from July 24, 2018)

**Fig. 3.** Flop at 37 °C of C₆NBD-PG (0.5 mol % of total PL) in proteoliposomes consisting of E. coli phospholipid extract and KcsA tetramer at molar ratios with respect to PL-Pi of 1:2000 (triangles, n = 2, the error bars showing the variation) and 1:1000 (diamonds, n = 2, single data points), and in proteoliposomes without protein (circles, n > 3, error bars indicate the S.D.). The curves represent the best fit of the data according to Equation 4. The inset shows a fragment of a Coomassie-stained SDS gel loaded with KcsA before (lane 1, 1.3 μg of protein from a stock solution) and after (lane 2, 2.4 μg of protein) reconstitution in proteoliposomes composed of E. coli PL, at a 1:1000 tetramer/PL-Pi molar ratio.

| Protein/peptide | Molar ratio | Proteoliposomes 37 °C | LUVETs 37 °C | LUVETs 25 °C |
|-----------------|-------------|-----------------------|----------------|----------------|
| No protein/peptide | 0 | 0.056 | 0.050 | 0.031* |
| WALP23 | 1:1000 | 0.35 | 0.80 | 0.17** |
| WALP23 | 1:250 | ND | ND | 0.6* |
| H₁ | 1:125 | ND | ND | 1.61 |
| Lep | 1:1000 | 0.16 | 0.21 |
| KcsA (tetramer) | 1:1000 | |

* Data are from Ref. 12.
** ND, not determined.
† Data are from Ref. 11.
KcsA. Addition of ATP did not initiate flip mediated by MsbA (black symbols) nor did it influence flip in vesicles without protein as expected. In addition, no significant protein-induced flip of C6NBD-phosphatidylethanolamine was observed in TLE vesicles (not shown), irrespective of the presence of ATP, which was hydrolyzed at a rate of 10.4 ± 3.0 nmol/min·mg protein−1 during the assay (i.e. at 37 °C), in agreement with data reported (26). These data indicate that MsbA by itself is not capable of ATP-dependent phospholipid translocation and that not all E. coli inner membrane proteins induce passive phospholipid translocation.

So far, it has been demonstrated that phospholipid translocation is facilitated by a subset of proteins with α-helical membrane-spanning segments. To investigate further whether the α-helical stretches are necessary for translocation, the β-barrel protein OmpT was also tested. The protected pool of C6NBD-PG in the reconstituted proteoliposomes containing OmpT was ~37% and ~50% in the control vesicles without OmpT. Recovery of phospholipid and protein was somewhat lower than for the other proteins, around 20%. In the presence of OmpT, we observed a slightly higher offset of flop, which is most likely due to dithionite permeation. The permeance (Fig. 5 inset, lane 2) of OmpT in a model membrane did not result in increased flop of C6NBD-PG (Fig. 5, diamonds) as compared with vesicles without OmpT (Fig. 5, circles). This confirms the observation that facilitating flip-flop is not a general property of membrane proteins and is consistent with the hypothesis that phospholipid translocation is mediated by transmembrane α-helices.

**DISCUSSION**

The goal of the present study was to investigate whether phospholipid translocation could be induced by the mere presence of transmembrane proteins. We therefore selected a subset of membrane proteins amenable to reconstitution from detergent solution into proteoliposomes. It was shown that the method of reconstitution results in vesicles in which phospho-

![Image](70x552 to 294x737)

**Fig. 4.** Flip of C6NBD-PG (0.1 mol %) at 37 °C in proteoliposomes composed of TLE and MsbA (1:1000 molar ratio with respect to PL-Pi), incubated with (black triangles) or without (open triangles) 2 mM ATP/ Mg, and in proteoliposomes without protein (circles, data ± ATP were averaged). Fluorescence measurements were performed in 50 mM HEPS, 50 mM NaCl, pH 7.5. Data points with MsbA represent average values from three independent experiments performed in duplicate ± S.D., and the curves represent the best fit of the data according to Equation 4. The inset shows a part of a Coomassie-stained SDS gel showing the E. coli inner membrane protein MsbA, before (lane 1, 0.9 μg of protein from the stock solution) and after reconstitution (lane 2, 1.2 μg of protein) in proteoliposomes composed of E. coli PL, at a 1:1000 molar ratio with respect to total phospholipid.

![Image](329x332 to 551x470)

**Fig. 5.** Flip of C6NBD-PG (0.5 mol %) at 37 °C in proteoliposomes composed of TLE with (diamonds, 1:1000 protein/phospholipid molar ratio) or without (circles) OmpT. Data of two independent experiments performed in duplicate are shown, and the curves represent the best fit of the data according to Equation 4. The inset shows a part of a Coomassie-stained SDS gel with OmpT, before (lane 1, 0.5 μg of protein from stock solution) and after (lane 2, 0.4 μg of protein) reconstitution.

![Image](325x554 to 555x737)

**Fig. 6.** Model for the helix-mediated translocation of PL. A, single membrane-spanning peptides interact dynamically with the membrane and expose the maximal amount of surface to the membrane interior creating a potential flop site. B, helix-helix interaction may reduce the dynamic interaction with the membrane, which may reduce flop. Additionally, less helix surface is exposed to the lipids (C), and thus the amount of potential flop sites is reduced.

lipid flip-flop can be monitored, and that any residual detergent does not enhance phospholipid translocation.

We showed that the E. coli inner membrane protein leader peptidase, an essential protein involved in membrane biogenesis, induces phospholipid translocation. It has been suggested that in membrane biogenesis, lipid and protein assembly might be functionally coupled (38). Thus, one might speculate that these two transport processes converge at Lep. However, the present study indicates that translocation of phospholipids is not directly linked to the protein transport machinery, as the potassium channel KcsA also induces flop.

In studies with model peptides, we generally observed rapid flip of C6NBD-PG at low peptide/phospholipid molar ratios (see e.g. Fig. 1 and Ref. 11). Here we also show that a naturally occurring membrane-spanning helix, H1 of Lep, induces efficient translocation. This indicates that the specific structure of the model peptides with Leu-Ala repeats as a hydrophobic core is not a prerequisite for efficient phospholipid translocation, validating their use as representatives of membrane-spanning helices of proteins. These observations, taken together with the fact that leader peptidase facilitates translocation whereas its...
water-soluble P2 domain does not, indicate that flop is induced by the transmembrane segments.

The proteins Lep and KcsA, incorporated at similar protein/phospholipid molar ratios as the membrane-spanning peptides, show a moderate effect on phospholipid translocation, compared with the model TMHs. The method of vesicle preparation could have some effect on the flop rates, as demonstrated by the different flop rates induced by WALP23 in vesicles prepared by detergent removal and extrusion, but this effect alone cannot account for the differences between peptides and proteins observed in this study. We speculate that the model peptides, being single helices and typically smaller than 3 kDa, interact more dynamically with the lipid bilayer than membrane proteins, which span the membrane more often and are significantly larger. Therefore, the peptides may cause more disturbances in the lipid-helix interface. Additionally, helix-helix interactions in membrane proteins could effectively reduce the “surface” available as flop sites, as will be discussed shortly. Thus, the monomeric Lep could be expected to have more flop sites per TMH available than the tetrameric KcsA, and our data show that indeed Lep exhibits more translocating activity per monomer than KcsA. MabA, a dimer with a total of 12 helices packed in the membrane, did not induce phospholipid translocation, consistent with this idea.

Based on several observations outlined in the Introduction, MabA has been suggested to play a role in phospholipid transport. However, phospholipid translocation in the bacterial cytoplasmic membrane has been shown to proceed without an energy source (1, 39). Here we showed that MabA was vesicle-associated after reconstitution and that it hydrolyzed ATP in proteoliposomes, as was reported previously (26). Therefore, we consider it likely that MabA was functionally reconstituted. However, no MabA-mediated flip in the presence or absence of ATP was observed. Possibly, MabA needs a co-factor to be functional in phospholipid flip-flop. Alternatively, MabA could be involved in transport of (phospho)lipids to the outer membrane or in translocation/transport of LPS, as has been suggested previously (27, 40). The data presented here argue against MabA being a phospholipid flipase.

The E. coli outer membrane β-barrel protein OmpT did not induce flop in TLE vesicles, consistent with our finding that α-helical membrane-spanning segments facilitate phospholipid translocation and with the fact that under normal conditions glycerophospholipids are absent from the outer leaflet of the outer membrane. The lipid asymmetry, with LPS exclusively in the outer leaflet, has been suggested to be important to preserve the barrier function of the outer membrane (41). However, it should be noted that reconstitution of OmpT in a glycerophospholipid double layer may not adequately reflect the properties of this protein.

Helix-mediated passive phospholipid flip-flop is consistent with the reported lack of energy requirement and lack of sensitivity toward treatment with alkylating agents reported for this process in vitro in isolated inner membrane vesicles of E. coli (1). However, the induction of phospholipid flip-flop is restricted to a subset of membrane proteins. Based on our previous studies (12), we proposed that translocation of phospholipids occurs near a transmembrane α-helix, where a “flop site” is created by the helix through the disturbance of the lipid-lipid interactions. The present findings lead to a refined model depicted in Fig. 6. Membrane-spanning α-helical segments induce flop of phospholipids through flop sites, which occur near the helix through its dynamic interaction with the membrane (Fig. 6A). When more helices are assembled, both the surface available as a potential flop site (Fig. 6C) and the dynamics of the helices are reduced (Fig. 6B), resulting in less efficient flop. The presence of residual detergent in proteoliposomes may partially fill up the peptide-induced defects, resulting in lower flop rates.

To address the question whether phospholipid translocation in E. coli in vivo could be accounted for by the presence of TMHs of integral membrane proteins, the number of TMHs per phospholipid in the E. coli inner membrane was previously estimated to be 1.9. Based on this ratio, the peptide-mediated translocation half-time at low peptide concentrations measured in model systems was extrapolated to that in the E. coli IM. However, the membrane protein Lep is substantially less efficient than a peptide in inducing flop, indicating that the above extrapolation may have overestimated the rate of helix-induced flop rate in the E. coli IM. When the observed translocation half-time of C6-NBD-PI induced by reconstituted Lep is extrapolated to the expected TMH/lipid ratio of the inner membrane of E. coli, a translocation half-time of ~6 min could be expected, which is sufficient to sustain growth at a doubling time of ~30 min. This estimate does not take into account proteins like MabA that do not induce phospholipid translocation. On the other hand signal peptides, for example, of exported membrane proteins might induce additional phospholipid translocation before they are degraded. Finally, care should be taken when translocation rates of phospholipid analogues are compared with those of their natural counterparts. For example, Huijbregts et al. (1) demonstrated that flop of NBD-phosphatidylethanolamine in the E. coli inner membrane has a translocation half-time of around 7 min, while its natural homologue redistributes within 30 s (2). Marx et al. (5) and Colleau et al. (42) observed different flop rates for NBD-PL versus the spin-labeled phospholipid analogues in rat liver microsomes and erythrocyte membranes, respectively. The studies cited above suggest that flop of C6-NBD-PL generally proceeds at a somewhat lower rate. This is according to expectation, because the rather bulky, relatively hydrophilic NBD group has to cross the hydrophobic interior of the bilayer.

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