

Lipid-Protein Interactions Drive Membrane Protein Topogenesis in Accordance with the Positive Inside Rule*

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Transmembrane domain orientation within some membrane proteins is dependent on membrane lipid composition. Initial orientation occurs within the translocon, but final orientation is determined after membrane insertion by interactions within the protein and between lipid headgroups and protein extramembrane domains. Positively and negatively charged amino acids in extramembrane domains represent cytoplasmic retention and membrane translocation forces, respectively, which are determinants of protein orientation. Lipids with no net charge dampen the translocation potential of negative residues working in opposition to cytoplasmic retention of positive residues, thus allowing the functional presence of negative residues in cytoplasmic domains without affecting protein topology.

Membrane Protein Assembly

Membrane protein topogenesis, the process by which TM⁴ orientation of a membrane protein is determined, is directed by a combination of several factors, leading to a predictable final organization for many but not all membrane proteins (1, 2). Initial TM topology is determined by topogenic signals residing within the protein sequence (1, 3) and protein interaction with the ribosomal channel (4) and the translocon machinery (5). Final topology is determined after the completed polypeptide chain exits the translocon by internal protein-protein interactions and protein-membrane lipid interactions during folding into the final compact protein (6–8). Although TM organization of many membrane proteins is established, the process by which these domains achieve their final orientation with respect to the plane of the membrane bilayer is not fully understood.

The biogenesis of polytopic membrane proteins has been extensively reviewed (9, 10). The signal recognition particle binds to the N-terminal signal sequence of a nascent polypeptide as it exits the ribosome and directs the docking of the complex onto the membrane-integrated translocon via interaction with the signal recognition particle receptor. Translation proceeds with the N-terminal extramembrane domain exiting the translocon on either the cytoplasmic (*cis*) or opposite (*trans*) side of the membrane. The first TM translates into the translocon pore and then exits laterally into the hydrophobic core of the membrane bilayer. The next extramembrane domain generally exits the translocon on the opposite side of the membrane relative to the preceding extramembrane domain, followed by a repeat of the above cycle until the initial insertion and orientation of TMs are completed. Therefore, the side of the membrane through which the flanking extramembrane domains exit the translocon initially determines the orientation of each TM. This initial orientation is governed by interaction of the nascent polypeptide with the ribosomal and translocon channels and is largely determined by the “positive inside rule,” which is based on the statistically and biochemically verified observation that the overwhelming majority of extramembrane domains facing the cytoplasm carry a net positive charge in contrast to the *trans*-domains, which carry either no charge or a net negative charge (1).

TMs may adopt an initial topology by direct charge interactions of the protein with the translocon and ribosome; however, the contribution of these molecular machines to topological decisions is limited by time, the size of newly synthesized protein, and the effective size of the translocation pore, which is still matter of debate (5, 9). The molecular basis for the positive inside rule is not fully understood, nor is the dominant effect of positively over negatively charged residues in determining final orientation of TMs. The positive inside rule is not absolute because cytoplasmic residency of net negatively charged domains is observed (11, 12), but in domains containing both positive and negative residues, the retention potential of the former generally dominates over the translocation potential of the latter (1, 2). Negative residues exhibit significant translocation potential when in excess over positive residues (1), flank a TM of low hydrophobicity (13), or lie within six residues of the TM-aqueous interface (3).

Once the nascent chain exits the translocon, final topology and folding events are governed by interactions with molecular chaperones, interactions within the protein itself, and interactions between the protein and the lipid environment that result in a thermodynamically determined energy minimum for the system (8, 14). The membrane environment is a complex milieu composed of the hydrophobic lipid bilayer, the flanking hydrophilic lipid headgroups, and the interfacial region that bridges the membrane surface with the aqueous solution surrounding membranes (15). Therefore, the folding environment of a membrane protein is considerably more complex than that of a soluble protein due to the necessity to attain an energy minimum that satisfies a range of hydrophobic, hydrophilic, and

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⁴ The abbreviations used are: TM, transmembrane domain; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PGP, phosphatidylglycerophosphate; MGLcDAG, monoglucosyldiacylglycerol; DGLcDAG, diglucosyldiacylglycerol; PC, phosphatidylcholine.

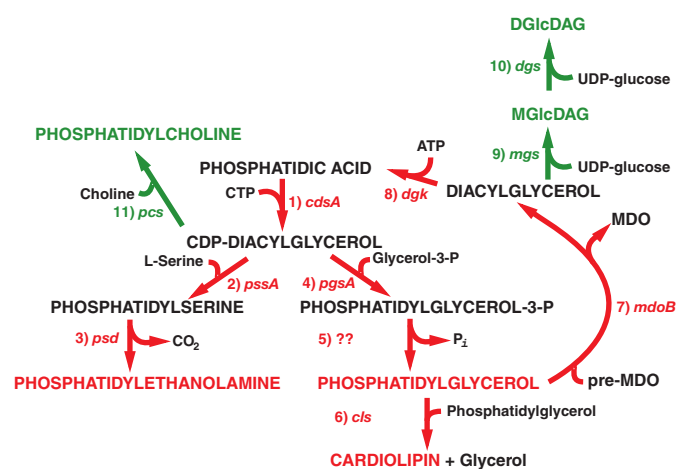


FIGURE 1. Pathway for synthesis of native and foreign lipids in *E. coli*. The following enzymes are indicated with their respective genes (from *E. coli* (17) unless indicated otherwise): 1, CDP-diacylglycerol synthase; 2, phosphatidylserine synthase; 3, phosphatidylserine decarboxylase; 4, PGP synthase; 5, PGP phosphatase; 6, CL synthase; 7, PG, pre-MDO (membrane-derived oligosaccharide) *sn*-glycerol-1-P transferase; 8, diacylglycerol kinase; 9, MGlcDAG synthase (*Acholeplasma laidlawii*) (19); 10, DGlcDAG synthase (*A. laidlawii*) (20); 11, PC synthase (*Legionella pneumophila*) (29). The X in phosphatidic acid is an OH and is in the position that changes depending on the downstream pathway. The lipids in black (~5% of total phospholipids) and red are native to *E. coli*. The lipids in green are foreign lipids introduced into *E. coli* carrying the indicated genes.

ionic interactions. The lipid bilayer has largely been treated as a static hydrophobic domain flanked by unspecified ionic groups much like a detergent micelle. However, biological membranes are highly dynamic structures made up of lipids with a broad spectrum of hydrophobic domains and headgroups that range from uncharged to zwitterionic to anionic. Because of the complexity of membrane protein assembly, only recently have the properties of the lipid bilayer been considered as a determinant of the final topological organization of membrane proteins. This review will focus on the role of lipid-protein interactions in determining the final organization of polytopic membrane proteins in bacteria. A more extensive review of this topic is available (16).

Engineering Changes in Membrane Lipid Composition

Because the overall lipid composition of most membranes remains relatively constant despite dynamic local changes in lipid composition, a role for lipid composition in determining membrane protein organization is not readily detected in wild-type cells. However, molecular genetic manipulation of membrane lipid composition (17) has revealed that the determinants of topological organization encoded into the amino acid sequence of membrane proteins are dependent on the host membrane lipid composition (6, 8, 11, 12). The engineering of *Escherichia coli* mutants with altered native phospholipid composition and the introduction of foreign lipids into these mutants provide powerful reagents to dissect the dependence of protein topology on the lipid environment. *E. coli* phospholipid composition (Fig. 1) varies from 70 to 80% PE, 20 to 25% PG, and 5 to 10% CL. Null mutants in the *pssA* gene are viable, have defects in cell division and secondary solute transporters, and lack amine-containing and zwitterionic lipids downstream of the left branch of the pathway (17). Null mutants in the *pgsA*

gene (18) are also viable, require second site repressor mutations for viability; show reduced rates of protein translocation across the inner membrane; and have <10% anionic phospholipids, which are mainly the precursors to PGP. Placing the *pssA* gene under the control of an inducible promoter allows synthesis and membrane assembly of a target membrane protein in the absence of PE, followed by induction of PE synthesis to study the effects of changes in the lipid environment post-assembly of protein *in vivo* (6, 8, 11). The foreign neutral lipids MGlcDAG (19) and DGlcDAG (20) and the zwitterionic lipid PC⁵ can be synthesized in PE-lacking *E. coli* by introducing the appropriate foreign genes. These foreign lipids correct many of the phenotypes of PE-lacking cells. This collection of “lipid reagents” has been essential to define specific roles for lipids in determining the organization and function of a subset of membrane proteins *in vivo*.

Lactose Permease as a Model for Studying Lipid-Protein Interactions

Lactose permease (LacY) of *E. coli* is a paradigm for secondary transporters that couple uphill movement of solutes across membranes to the membrane electrochemical potential (21). LacY has 12 TMs with its termini facing the cytoplasm (Fig. 2A). The cytoplasmic domains follow the positive inside rule, with the periplasmic domains being neutral or net negative. The crystal structure (22) and biochemical studies (23) demonstrate that the final structure results from the independent folding of the two halves of LacY into compact six-TM helical bundles connected by an extramembrane cytoplasmic domain (C6). Reconstitution of LacY into liposomes of total *E. coli* lipids results in native uphill energy-dependent transport and downhill energy-independent transport, whereas liposomes composed of PG and CL with or without PC support only downhill transport (24). The dependence on PE for full activity of LacY was demonstrated to be physiological by the lack of uphill transport in *E. coli* mutants (*pssA* null) lacking PE (25), which provided the rationale to investigate whether lipid composition affects protein structure.

Beginning with a topological model of LacY in PE-containing cells, which was validated by high-resolution structural analysis (22), the orientation of TMs with respect to the plane of the bilayer was determined based on the cytoplasmic or periplasmic residence of the extramembrane domains of LacY as a function of membrane lipid composition (Fig. 2A). The accessibility to a membrane-impermeable sulfhydryl reagent of single-cysteine replacements in the extramembrane domains of LacY in the membrane of whole or broken cells was used to determine the orientation of neighboring TMs (26). The surprising observation was that in cells lacking PE (Fig. 2B), the N-terminal six-TM bundle was completely inverted with respect to the C-terminal bundle and the membrane bilayer (6, 8), with TMVII now exposed to the periplasm (8). LacY is fully stable in this inverted configuration and exhibits only downhill transport of substrate, indicating that it is in a new compact folded state (6, 8). Even more remarkable is that synthesis of PE post-assembly of LacY (Fig. 2C) induces a near-complete inver-

⁵ P. Heacock, M. Bogdanov, and W. Dowhan, unpublished data.

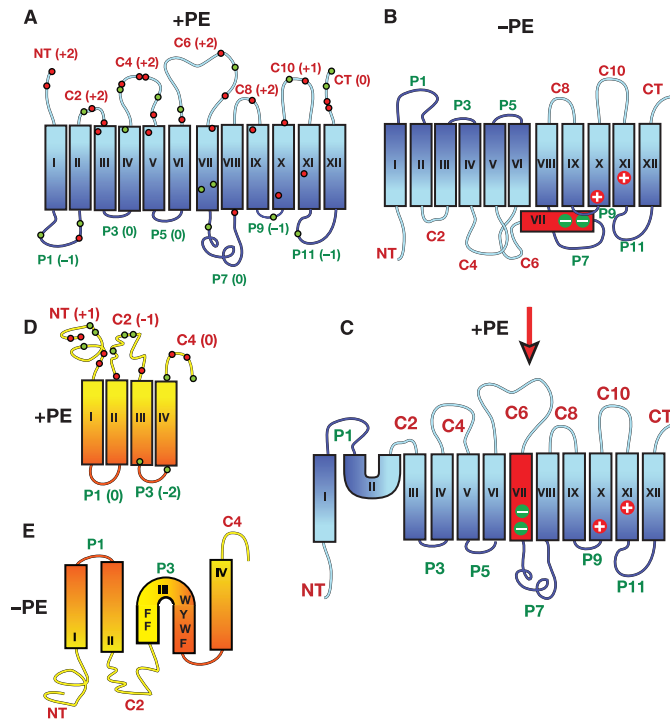


FIGURE 2. Topological organization of secondary transporters as a function of membrane lipid composition. The cytoplasm is at the top of each figure; TMs are noted by rectangles; NT and CT refer to the N and C termini, respectively; and extramembrane domains oriented to the cytoplasm (C) or periplasm (P) in PE-containing cells are indicated. A, LacY topology as determined in PE-containing (+PE) cells is depicted. Green (negative charge) and red (positive charge) dots indicate the approximate positions of charged residues with the net charge of each extramembrane domain noted next to the domain name. B, LacY topology as determined in PE-lacking cells is shown. The exposure of TMVII (red) to the periplasm results in the loss of salt bridges with TMX and TMXI. C, shown is LacY topology determined after induction of PE synthesis in cells where assembly of LacY initially occurred in the absence of PE. D, PheP and GabP topology (topology of the lipid-insensitive TMV to CT domains not shown) as determined in PE-containing cells is shown. The same nomenclature is used as in A. E, the topology of PheP and GabP assembled in cells lacking PE is shown. The high content of aromatic residues in TMIII is indicated.

sion of the N-terminal bundle, insertion of TMVII across the membrane, and regain of uphill transport (8). Therefore, final LacY topological organization is sensitive to the lipid environment during initial biosynthesis and to changes in the lipid environment after folding into a compact structure. When reconstituted into liposomes lacking other proteins, LacY topology and function are similarly dependent on the lipid environment irrespective of whether LacY was purified from PE-containing or PE-lacking cells (24), which strongly indicates that lipid-protein interactions are a determinant of TM orientation independent of other cellular components.

Phenylalanine permease (PheP) (11) and γ -aminobutyrate permease (GabP) (12) of *E. coli* are also dependent on PE for full uphill transport function and the topological orientation of the N-terminal two-TM helical hairpin (Fig. 2, D and E). The native topology and function of PheP are also restored by post-assembly synthesis of PE. In addition, several other secondary transporters of *E. coli* fail to carry out uphill transport in PE-lacking cells.⁶ Therefore, a requirement for PE at least for several sec-

ondary transporters is physiologically important for both structure and function.

Nature of Lipids That Support Native Topology

What features of the lipid bilayer control integral membrane protein orientation? When synthesized in *E. coli*, several foreign lipids support the wild-type topology of LacY in the absence of PE. Cells expressing MGLcDAG to ~30–40% of total lipid support both uphill transport by LacY and the wild-type orientation of all TMs (19). However, there appear to be subtle differences from LacY assembled in PE-containing cells in the folding of or possibly the solvent accessibility of some periplasmic domains. Introducing genes that result in near-complete conversion of MGLcDAG to DGLcDAG also results in the native topology of LacY but with only downhill transport function (20). Finally, introducing PC to 70% of total lipid results in uphill transport and native topology.⁷ Although reconstitution of LacY in PC-containing liposomes results in native topology, such liposomes support only downhill and not uphill transport (24). What emerges from these studies is a clear picture of lipid properties that support native topology, but a clear understanding of the properties of lipids that support uphill transport remains unclear. The foreign lipids and PE have diverse physical and chemical properties ranging from neutral (glycolipids) to zwitterionic (PE and PC), to H-bonding capability (all but PC), to bilayer (PC and DGLcDAG) and non-bilayer (PE and MGLcDAG) propensities. However, the common feature of these lipids is the ability of zwitterionic and neutral lipid headgroups to dilute the high negative surface charge density of a bilayer composed of PG and CL. Therefore, the ratio of anionic lipids to lipids with no net charge appears to be a primary determinant of membrane protein topology.

Lipid-sensitive Topogenic Determinants

What topogenic signals within a protein sequence make a protein sensitive to membrane lipid composition during initial protein assembly as well as to changes in lipid composition after synthesis and stable assembly in the membrane? Early studies investigated the simultaneous effect on TM orientation of altering the ratio of zwitterionic to anionic phospholipid content of *E. coli* and the number of positive amino acids in potential cytoplasmic domains of a bitopic membrane protein (27). Cytoplasmic retention of an extramembrane domain was increased with increasing net positive charge of the domain when the anionic lipid content of the membrane was low and with increasing anionic lipid content of the membrane when the net positive charge of the domain was low. Thus, a simple charge interaction between positive amino acids and negative lipid headgroups was proposed to determine topology.

Inspection of the lipid-sensitive extramembrane domains of LacY shows that the cytoplasmic domains all carry a net positive charge and that the periplasmic domains carry either a net zero or negative charge. Based on the above studies, a dramatic increase in the anionic lipid headgroup content in PE-lacking membranes should favor a native rather than an inverted topology for LacY. However, the earlier experiments did not con-

⁶ M. Bogdanov, H. Vitrac, and W. Dowhan, unpublished data.

⁷ M. Bogdanov and W. Dowhan, unpublished data.

sider the influence of negatively charged residues on topology as a function of lipid composition. The translocation potential of negative residues is much weaker than the cytoplasmic retention potential of positive residues (2). Several negative residues within six residues of a TM end (3) or flanking a TM of low hydrophobicity (13) are usually required to override the retention potential of a single positive residue. In fact, the lipid-sensitive cytoplasmic domains of PheP and GabP actually carry a net negative charge (11, 12) yet reside on the *cis*-side of the membrane in wild-type cells. Dissecting the features of these permeases that necessitate PE for native topological orientation provided new insight into the role of lipids in establishing membrane protein topology.

These permeases contain two features that were postulated to result in a dependence on native lipid composition for proper TM orientation (8). First, the inverted structures display a topologically flexible hinge region between the lipid-sensitive and lipid-insensitive domains, resulting in one fewer TM in the inverted structure (Fig. 2). Second, the cytoplasmic domains that become periplasmic contain a high number of negative residues. For LacY assembled in PE-lacking cells, TMVII no longer spans the membrane and is most likely exposed to the periplasm as an extramembrane domain. TMVII is of low hydrophobicity due to two Asp residues, which are stabilized in the membrane of wild-type cells through salt bridges to TMX and TMXI (21). Increasing the hydrophobicity of TMVII by replacing one Asp residue with Ile prevents TMVII from being released into the periplasm in PE-deficient cells and blocks the inversion of the N-terminal bundle. TMVII inserts back into the membrane upon reorganization of LacY after synthesis of PE (8). Reorientation of LacY by post-assembly synthesis of PE does not include TMI and results in an apparent mini-loop structure for TMII (Fig. 2C). TM switching appears to rely on the intrinsic structural flexibility provided by TMI and TMVII as mobile molecular hinges, which are necessary for TM rearrangement in response to changes in the lipid environment. Therefore, a thermodynamic balance exists between the apparent lower energy minimum that drives inversion of the N-terminal bundle in PE-lacking cells and the energy cost of exposing a TM to solvent (16). TMIII in PheP and GabP appears to form a mini-loop that does not traverse the membrane (Fig. 2, D and E) and thus acts as a similar molecular hinge (11, 12). TMIII in PheP and GabP is highly enriched in aromatic amino acids that are normally found at the membrane-solvent interface, which may stabilize TMIII as a mini-loop near the membrane surface.

Altering the net charge of the cytoplasmic domains of these permeases provided a more precise understanding of lipid-protein interactions in establishing topology (8). Inversion of LacY in PE-lacking cells was prevented by increasing the net charge of the cytoplasmic surface of the N-terminal bundle by +1 in a position- and sequence-independent manner (*i.e.* in domain C2, C4, or C6). Making compensating changes in charged residues that did not alter net charge did not prevent inversion of topology in PE-lacking cells. Finally, introducing acidic residues on the normally cytoplasmic face of LacY induced inversion in PE-containing cells but required making all three cytoplasmic domains net -2 . Therefore, negatively charged residues contribute to orientation in a cooperative and cumulative manner

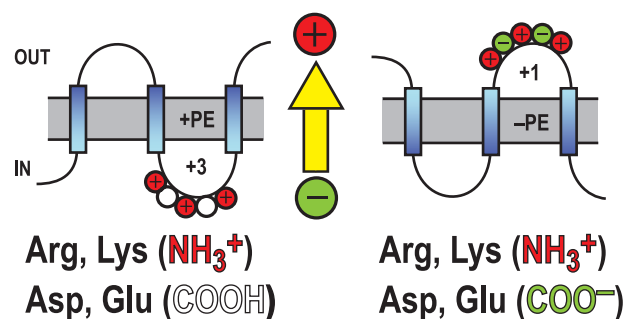


FIGURE 3. PE and the positive inside rule. A cytoplasmic domain containing both negatively and positively charged amino acids is shown. PE dampens the translocation potential of negative residues in favor of the cytoplasmic retention potential of positive residues (*left*). The effective net positive charge (+3) of the domain is sufficient for cytoplasmic retention. Without PE, the net positive charge is +1, which is insufficient for cytoplasmic retention with the possibility that negative residues are dominant over positive residues in the absence of PE (*right*). The protonmotive force (*arrow*) positive outward determines domain directional movement depending on the domain effective net charge as influenced by the lipid environment. Induction of PE synthesis post-assembly of a protein reverses the effective net charge and drives TM flipping (8).

to trigger an inversion of the N-terminal bundle but are significantly less potent in PE-containing than in PE-lacking cells. Similar cumulative charge effects were seen for PheP.⁷

Although the positive inside rule is well accepted, it is not clear if or how positively charged residues exert their effect on topology after a protein leaves the translocon, why positive residues dominate over negative residues as orientation determinants, and what other cellular factors govern final topological decisions. The above results support a role for PE (and presumably other lipids with no net charge) in strongly attenuating the translocation potential of acidic residues located in cytoplasmic domains, resulting in a strengthening of the contribution of positive residues to the retention potential, and provide a molecular basis for the weakness of negative residues as translocation signals when flanked by positive residues (Fig. 3). In the absence of PE, negative residues exert their full translocation potential and result in translocation of a domain that exhibits a lower effective net positive charge. This allows for the presence of significant numbers of negatively charged amino acids in cytoplasmic domains for functional and structural reasons without affecting final topological organization. The complementary effect of increasing the net positive charge on the protein or diluting the negative charges of the lipid headgroups to support cytoplasmic retention demonstrates an interaction between proteins and lipids in determining final topology.

Conclusions

Simply changing the lipid composition of the membrane either before or after membrane protein insertion can reverse the topology of a polytopic membrane protein. The results clearly demonstrate that the lipid composition is a determinant of TM orientation and challenge the dogma that once TM orientation is established during assembly, it is static and not subject to change. Therefore, proteins and lipids have co-evolved so that protein sequence determines topology, but the sequence is written for a specific lipid environment. Lipid-protein charge interactions during membrane protein biogenesis or after stable assembly can contribute to folding anomalies induced by

either minor sequence perturbations or major changes in the lipid compositions of membrane microdomains in inherited and non-inherited topological disorders (28).

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