Exploring the Interplay of Lipids and Membrane Proteins

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Exploring the Interplay of Lipids and Membrane Proteins

Candan Ariöz

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Aileme,
(To my family)

Every stormy night has a sunny morning. Never lose hope…

Abstract

The interplay between lipids and membrane proteins is known to affect membrane protein topology and thus have significant effect (control) on their functions. In this PhD thesis, the influence of lipids on the membrane protein function was studied using three different membrane protein models.

A monotopic membrane protein, monoglucosyldiacylglyecerol synthase (MGS) from *Acholeplasma laidlawii* is known to induce intracellular vesicles when expressed in *Escherichia coli*. The mechanism leading to this unusual phenomenon was investigated by various biochemical and biophysical techniques. The results indicated a doubling of lipid synthesis in the cell, which was triggered by the selective binding of MGS to anionic lipids. Multivariate data analysis revealed a good correlation with MGS production. Furthermore, preferential anionic lipid sequestering by MGS was shown to induce a different fatty acid modeling of *E. coli* membranes. The roles of specific lipid binding and the probable mechanism leading to intracellular vesicle formation were also investigated.

As a second model, a MGS homolog from *Synechocystis* sp. PCC6803 was selected. MgdA is an integral membrane protein with multiple transmembrane helices and a unique membrane topology. The influence of different type of lipids on MgdA activity was tested with different membrane fractions of *Synechocystis*. Results indicated a very distinct profile compared to *Acholeplasma laidlawii* MGS. SQDG, an anionic lipid was found to be the species of the membrane that increased the MgdA activity 7-fold whereas two other lipids (PG and PE) had only minor effects on MgdA. Additionally, a working model of MgdA for the biosynthesis and flow of sugar lipids between *Synechocystis* membranes was proposed.

The last model system was another integral membrane protein with a distinct structure but also a different function. The envelope stress sensor, CpxA and its interaction with *E. coli* membranes were studied. CpxA autophosphorylation activity was found to be positively regulated by phosphatidylethanolamine and negatively by anionic lipids. In contrast, phosphorylation of CpxR by CpxA revealed to be increased with PG but inhibited by CL. Non-bilayer lipids had a negative impact on CpxA phosphotransfer activity.

Taken together, these studies provide a better understanding of the significance of the interplay of lipids and model membrane proteins discussed here.

List of Publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **C Ariöz**, W Ye, A Bakali, C Ge, J Liebau, H Götzke, A Barth, L Mäler, Å Wieslander. (2013) Anionic lipid binding to the foreign protein MGS provides a tight coupling between phospholipid synthesis and protein overexpression in *Escherichia coli*. *Biochemistry*. **52** (33): 5533-5544

II. **C Ariöz**, H Götzke, L Lindholm, J Eriksson, K Edwards, DO Daley, A Barth, Å Wieslander. (2014) Heterologous overexpression of a monotopic glucosyltransferase (MGS) induces fatty acid remodeling in *Escherichia coli* membranes. *BBA-Biomembranes*. *In press*

III. TT Selão, L Zhang, **C Ariöz**, Å Wieslander, B Norling. (2014) Subcellular localization of monoglucosyldiacylglycerol synthase in *Synechocystis* sp. PCC6803 and its unique regulation by lipid environment. *PLoS ONE*. **9** (2): e88153

IV. R Keller*, F Stenberg-Bruzell*, M Burstedt*, **C Ariöz**, D Wikström, A Kelly, Å Wieslander, DO Daley, S Hunke. (2014) The *Escherichia coli* envelope stress sensor CpxA can sense changes in lipid bilayer properties. *Manuscript*

* These authors contributed equally

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Additional publications:

V. L Lindholm, **C Ariöz**, M Jawurek, J Liebau, L Mäler, C von Balmoos, A Barth, Å Wieslander. (2014) Effect of lipid bilayer properties on the photocycle of green proteorhodopsin. *Submitted*

VI. **C Ariöz***, P Uzdavinys*, O Beckstein, Å Wieslander, D Drew. (2014) The regulation of NapA activity by membrane lipid properties. *Manuscript*

* These authors contributed equally

Contents

Abbreviations

AD93	*Escherichia coli* cell line lacking PE lipid
CFAs	Cyclopropanated fatty acids
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CL	Cardiolipin (diphosphatidylglycerol)
CMC	Critical micelle concentration
CpxA	The envelope stress sensor of *Escherichia coli*
Cryo-TEM	Cryo-Transmission Electron Microscopy
DAG	Diacylglycerol
DDM	n-Dodecyl-β-D-maltoside
DHPC	1,2-Dihexanoyl-sn-Glycero-Phosphocholine
DMPC	Dimyristoyl-sn-Glycero-3-Phosphocholine
FT-IR	Fourier Transform Infrared Spectroscopy
GalDAG	Monogalactosyldiacylglycerol
GalGalDAG	Digalactosyldiacylglycerol
GFP	Green fluorescent protein
GlcDAG	Monoglucosyldiacylglycerol
GlcGlcDAG	Diglucosyldiacylglycerol
H_I	Normal hexagonal
H_{II}	Inverted hexagonal
IM	Inner membrane
L_α	Liquid-crystalline lamellar
LFM	Lipid-Fishing method
LPS	Lipopolysaccharide
MgdA	*Synechocystis* sp. PCC 6803 Monoglucosyldiacylglycerol synthase
MGS	*Acholeplasma laidlawii* Monoglucosyldiacylglycerol synthase
OD_{600}	Optical density at 600 nm
OM	Outer membrane
OMP	Outer membrane protein
PDB	Protein data bank (URL: http://www.rcsb.org/pdb/)
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PM	Plasma membrane
SFAs	Saturated fatty acids
SQDAG	Sulfoquinovosyldiacylglycerol
TLC	Thin layer chromatography
TM	Thylakoid membrane
TM	Transmembrane
UFAs	Unsaturated fatty acids
W3899	Mother cell line of AD93 mutant

1. Introduction

In the time of the ancient Byzantine Empire, trading with merchants from Europe and Asia was important for maintaining the comfort of the Byzantinians who had lived inside the great Theodosian wall. Besides providing the *goods* for the Byzantine population, defending the empire from the endless attacks of outsiders was of primary significance. Theodosian walls were built in the 5th century to protect the city from the sieges and it contained 50 gates constructed for communication with merchants or visitors from distant lands. If we can visualize the *cell* as the *Byzantine Empire*, the Theodosian land walls could be related to the biological membranes surrounding the cell components. Just like the *Byzantine* people, a cell needs to be protected against the fluctuations in the outer environment, but it also requires communication with its surrounding through gates. These gates could be considered as membrane proteins and the bricks of the walls as membrane lipids. The types of the interaction of the bricks with the gates define the strength of the walls and thus the types of both components of the wall have to be carefully chosen.

Biological membranes are composed of various types of lipids and membrane proteins. Both components communicate with each other strongly and any changes in one affects the other. The interplay of membrane lipids and proteins is evidenced by the tightly-associated lipids in the structures of membrane proteins. For years, membrane lipids were thought of as a passive solvent but nowadays it has been clearly understood that they can actually influence the structures of membrane proteins and thus have an effect on their functional roles in the membrane [1]. The physicochemical state of a biological membrane is greatly influenced by its lipid composition and properties such as curvature, fluidity, charge distribution, membrane thickness and hydrophobicity are shaped by the individual lipid molecules [2, 3]. These modifications could vary from species to species and could also depend on the specific requirements brought by the individual membrane proteins. Understanding the relationship between lipids and membrane proteins could help us to gain further insight into the mechanisms of certain cellular events, such as molecular sorting, transport between organelles or intracellular/extracellular vesiculation *etc*., that take place in the bilayer.

The *objectives* of this PhD thesis were to characterize the types of lipids that interact with three different membrane proteins (MGS, MgdA and CpxA) and understand the significance of these lipids on protein functions. The first part of this thesis briefly summarizes the background for membrane lipids, membrane proteins, lipid-protein interactions and approaches used during the work intended in this thesis. The second part contains a summary of the papers that this thesis is based on.

2. Biological Membranes

The general description of a biological membrane is given as "A defined boundary between the cellular components and extracellular medium, which protects the cell from dangerous fluctuations in order to keep homeostasis in the cell" [4, 5]. Membranes not only define a simple boundary but also create domains in which certain cellular activities can be carried out in a segregated fashion in order to make them more efficient [6, 7]. Membranes are dynamic structures with a constant activity at their interfaces. Cells need to transport both hydrophilic and hydrophobic molecules across membranes so that all vital processes (maintenance of electrochemical gradient, nutrition, ATP synthesis, keeping homeostasis *etc*.) can take place. During all transport events, the selectivity is achieved by the molecular gates of the bilayer: *membrane proteins* (**Figure 1**).

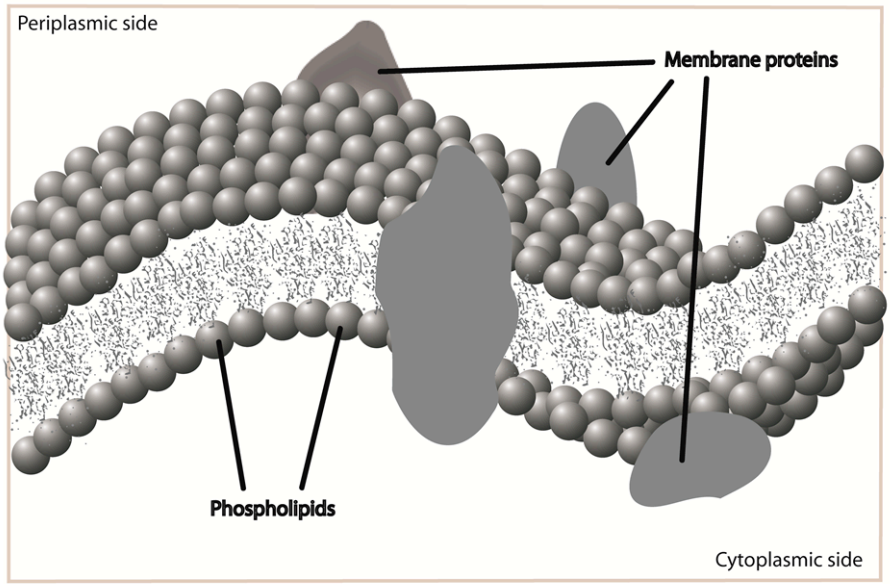

Figure 1. The biological membrane. According to the fluid mosaic model, a biological membrane is composed of membrane proteins embedded into a sea of lipid molecules. Phospholipid headgroups interact with polar residues of proteins and hence an interplay between two species generates a dynamic but well-sealed hydrophobic barrier.

For years, lipids were believed to act as a rather passive solvent for membrane proteins but recent studies indicate that they have significant roles in cellular processes [7]. For example, the water-lipid interface is a rough surface and lipid headgroups create an active interface that can change the concentrations of charged molecules or ions close to the membrane surface [2].

Any change in the surface charge is sensed by the membrane-associated or embedded proteins so that their activities are regulated by membrane lipids. It should however be noted that lipid-protein interactions are not limited to the bilayer surface. The interactions taking place at the lipid-protein interfaces are also important for the regulation of bilayer functions. At lipid-protein interfaces amino acid side chains will require the sealing of lipids to create a well-sealed hydrophobic barrier. Both partners are observed to affect each other [2, 8] and this interactive relation causes many significant cellular events [7].

2.1. Membrane Lipids

Lipids have distinct features that make them a better choice of building blocks for the construction of biological membranes. First of all, they are found at the most reduced state of the carbon atom so that their oxidation releases a great amount of energy. This feature enables the cell to store its energy in lipids and use it later for energy requiring processes. Another interesting feature arises from their chemical structure. Membrane lipids are amphiphilic molecules that have hydrophilic headgroups and hydrophobic acyl chains. This property enables them to self-assemble in an aqueous environment and thus segregates the cell from the outer environment. Additionally, this feature also brings the advantage to self-assemble membrane proteins/enzymes into domains. This is significant for achieving the highest enzymatic activity without any leakage of the enzymatic products. A third role of lipids is in signal transduction and molecular recognition processes [7]. The degradation products of some membrane lipids are reported to act as first and second messengers [9]. Lipids also play roles in cellular processes such as energy transduction, cellular trafficking, endocytosis/exocytosis, translocation of membrane proteins across membranes and even cellular defense mechanisms [7, 10-12].

Eukaryotic organisms dedicate a significant proportion of their genomes (5%) to lipid metabolism and its homeostasis [7, 10]. A rough estimation is that these genes are responsible for several hundred thousand distinct lipid species. However, it is still uncertain why eukaryotic cells devote 5% of their genome to create such a large repertoire and what advantages they gained over prokaryotes from this. A complete understanding about the usage of such an enormous number of lipid molecules remains elusive.

To produce such a great collection, every organism modifies lipid headgroups and acyl chains with respect to its *milieu* and metabolic processes [13, 14]. Since it has been shown that lipids can regulate the activities of membrane proteins, further knowledge about the organisms and their mem-

branes is required in order to understand this interplay. The model organisms studied in this thesis and their membranes are described in the next section.

2.1.1. Escherichia coli

Escherichia coli (*E. coli*) is the most common model organism studied so far and its metabolic pathways are well defined [15]. It is a gram-negative, facultative anaerobic, rod-shaped bacterium, which is 2 μm in length and 0.5 μm in diameter. This enterobacterium is known to reside in the lower intestines of warm-blooded organisms (endotherms) and be capable of adapting to changing environments with its dynamic cell envelope. This envelope consist two distinct membranes: an *inner membrane* and an *outer membrane*. These two membrane systems are separated by the periplasm containing the peptidoglycan layer. The outer membrane is an asymmetrical bilayer made up by phospholipids and lipopolysaccharides (LPS) in the inner and outer leaflets, respectively. Membrane proteins of the outer membrane span the membrane as amphipathic β-strands that fold into cylindrical β-barrels with a hydrophilic core and hydrophobic exterior that is exposed to lipid molecules [16-18]. The outer membrane acts as a selective barrier for nutrients or hydrophilic solutes and protects the bacteria from toxic compounds. Selectivity is achieved by the outer membrane protein complexes (OMPs) or *porins* which allow passage of molecules with molecular masses up to ~600 Da [17, 19].

Inner membranes, are phospholipid bilayers with proteins spanning the membrane (approximately 8 nm) with their hydrophobic α-helices [18]. The inner membrane is composed of ~40% (wt/wt) phospholipids (~ 16,000,000 molecules) and ~60% (wt/wt) proteins (~ 200,000 molecules) (http://ccdb.wishartlab.com). The phospholipid composition is ~75-80% phosphatidylethanolamine (PE, zwitterionic, non-bilayer prone), ~15-20% phosphatidylglycerol (PG, anionic, bilayer prone) and ~2-5% cardiolipin (CL, anionic, mainly bilayer prone) [20]. The physicochemical properties of these lipids will be discussed later in detail. However it should be noted that the shapes of the phospholipids have a deep impact on the physicochemical characteristics of the bilayer, which in turn affects the functions of membrane proteins [20, 21].

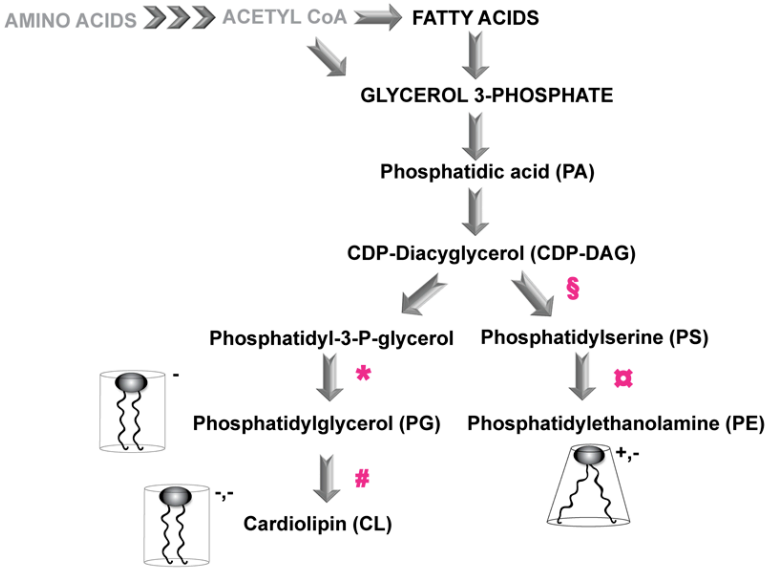

Figure 2. Phospholipid synthesis in *E. coli*. Phospholipids are constituted by fatty acids and glycerol backbones. Both building blocks are synthesized from acetyl coA, which is derived from carbon precursors (e.g. amino acids) in growth medium. Different phospholipids are produced via steps catalyzed by different enzymes: PE by Phosphatidylserine synthase-PssA (§) and phosphatidylserine decarboxylase-PSD (¤), respectively; PG by phosphatidylglycerol synthase-PgsA (*) and CL by cardiolipin synthase-ClsA (#). *E. coli* inner membrane composition differs at different growth conditions but usually accepted to be phosphatidylethanolamine ≈70-80%; phosphatidylglycerol ≈15-20% and cardiolipin ≈2-5%. The cardiolipin content usually increases when cells enter stationary phase. Additionally, CL levels are also elevated during the overexpression of MGS protein in *E. coli*.

PE-minus Strain (AD93)

PE has a headgroup with smaller cross-sectional area compared to its acyl chains thus forms an inverted hexagonal (H_{II}) phase. H_{II} phase lipids form high local curvatures and they evoke lateral pressure with their insertion into a bilayer with other lipids. Probably the lateral pressure created stabilizes membrane proteins to find the correct conformation [22-24].

To study the influence of lipids on membrane protein topology and function, a PE-knockout strain of *E. coli* was created by *Dowhan* and coworkers. This mutant was generated by the inactivation of the gene that encodes phosphatidylserine synthase (PssA). This is a membrane associated enzyme responsible for a committed step in PE synthesis [25] (**Figure 2**). Biochemical studies also validated the absence of PE lipid in the membrane. However, lacking a major lipid in the membrane has dramatic consequences for AD93 cells. First of all, the growth rate is decreased compared to its mother type

W3899 and the presence of divalent ions (Mg^{2+}, Mn^{2+}) is required for growth. Some lipids are known to be capable of changing the packing properties (shapes) in the presence of divalent ions and thus form hexagonal phases. In the AD93 strain, the lipid composition was observed to be ~60-70% PG and ~30-40% CL when cells are grown with 20 mM Mg^{2+}. The majority of the membrane seems to be composed of *bilayer-prone* lipids, however CL changes its shape in the presence of Mg^{2+} ions from *bilayer-prone* to *nonbilayer-prone* and replaces PE lipid. Nevertheless, these modifications do not fulfil PE absence and growth rates are really poor even the growth medium is supplemented with 20 mM Mg^{2+}. A replacement of another non-bilayer-prone lipid from *Acholeplasma laidlawii* (GlcDAG) was observed to improve the crippled growth of AD93 strain [20].

2.1.2. Acholeplasma laidlawii

Acholeplasma laidlawii (*A. laidlawii*) belongs to the family of Mollicutes (Mycoplasmas), which are considered to be the simplest self-replicating organisms. They are defined as cell wall-less, semiobligative-parasites living in animals, plants and microorganisms. *A. laidlawii* lacks many biosynthetic and degredative pathways, and it is able to synthesize only saturated fatty acids when required nutrients and supplements are added to growth medium [26]. This organism is incapable of synthesizing unsaturated lipids due to the lack of an enzyme. *A. laidlawii* has eight different glycerolipids in its membrane and these lipids were observed to have distinct physicochemical properties. The major lipids found in *A. laidlawii* membranes are GlcDAG and GlcGlcDAG synthesized by monoglucosyldiacylglycerol synthase (MGS) and diglucosyldiacylglycerol synthase (DGS), respectively. GlcDAG and GlcGlcDAG lipids have significant roles in determining the phase behaviour of *A. laidlawii* membranes [24, 27, 28]. The genes for MGS and DGS proteins were introduced previously into *E. coli* cells in order to understand their role in phase behaviour.

2.1.3. Synechocystis sp. *PCC6803*

Synechocystis sp. PCC6803 (also referred to as *Synechocystis*) is a cyanobacterium living in fresh water and is considered as an evolutionary midpoint between bacteria and plants. As a prokaryote, this organism lacks differentiated organelles but retains an advanced intracytoplasmic membrane system (so-called *thylakoid membranes*) in addition to its plasma membrane [29]. Thylakoid membranes are the main sites for energy transduction, photosynthesis and respiration [30]. Identification of the photosystem components and their metabolic regulation has been studied extensively at the proteomic level [31-33], however much less data is known about lipid synthesis.

Like all photosynthetic organisms, *Synechocystis* membranes contain mostly *glycolipids* and *galactolipids* in order to decrease the dependency on phosphate in lipid prodction. In cyanobacteria, monoglucosyldiacylglycerol (GlcDAG) is formed with the glucose transfer from UDP-glucose to diacylglycerol (DAG) by a glycosyltransferase termed as MgdA (also referred as *Syn*MGS). In comparison to *A. laidlawii* MGS, MgdA is an integral membrane protein having a single Rossman fold domain organization (GT-A) and does not appear to be affected by bilayer properties (Paper III). GlcDAG is then epimerized into galactosyldiacylglycerol (GalDAG) by an uncharacterized epimerase. The transfer of another galactose to GalDAG is achieved by a *Synechocystis* digalactosyldiacylglycerol synthase, Slr1508. This pathway has been conserved through evolution from bacteria to plants [34].

2.2. Physicochemical Properties of Lipids

Lipids not only define the hydrophobic barriers of biological membranes but also the physicochemical properties. For a functional membrane, two criteria should be fulfilled: maintenance of a *crystalline phase* and *low permeability* [35]. Structural differences in lipids are known to affect their physicochemical properties and hence the broad lipid diversity should not be seen as a coincidence. Many strategies exist to diversify the lipid species in a biological membrane. Fatty acid and headgroup modifications are the most common strategies and are known as *homoviscous adaptation*. Bacteria can regulate the synthesis of new lipids but also modify the existing species in order to maintain the required membrane properties [13]. Some of the common modifications observed in bacteria are discussed below.

2.2.1. Fatty acid modifications

Fatty acids are the hydrophobic entities of a lipid molecule and usually have a broad range of diversity in the membranes. Fatty acids or acyl chains determine the viscosity of a biological membrane and influence its permeability.

Four types of fatty acids exist in bacterial membranes: saturated (SFA), unsaturated (UFA), branched-chain (BCA) and cyclopropanated (CFA). Saturated fatty acids, such as palmitic acid (16:0), are linear and pack tightly to form a bilayer with high phase transition and low permeability (**Figure 3**). Bacteria can introduce double bonds into growing fatty acids via FabB or FabZ enzymes, which results in a pronounced kink in the chain [13]. Because of this kink, acyl chains can not pack tightly hence the order of the bilayer is distrupted.

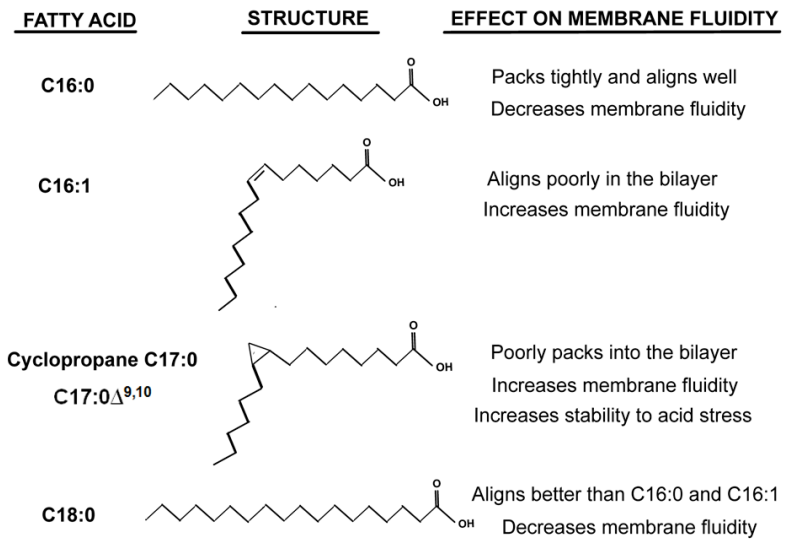

Figure 3. Different fatty acids and their effects on membrane properties. The kink brought by double bonds tends to induce a disorder when lipids are aligned in a bilayer. However, the cyclopropane ring creates more disorder, which disturbs acyl chain ordering of lipids and increases permeability to solutes. Adapted from ref [13].

Membranes with UFAs have lower transition temperatures and higher permeability. *Cis*-UFAs create membranes with lower phase-transition temperatures, increased fluidity and high permeability to solutes (less ordered) compared to *trans*-UFAs. However, few bacteria have evolved to convert *cis*-UFAs to *trans*-UFAs and generate membranes with higher phase-transition temperatures, increased rigidity and decreased permeability to solutes (more ordered). Besides unsaturation, branching of the acyl chains is another common modification in bacterial membranes. Branched-chain fatty acids create an effect similar to UFAs, which leads to altered membrane order. Since the added methyl group affects the alignment of acyl chains, the position is important to determine the effects on the bilayer. *Anteiso*-fatty acids have their methyl branch close to the mid-point of the acyl chain and promote a more fluid membrane compared to the bilayers with *iso* fatty acids (the methyl group is in the end of the acyl chain). Bacteria are known to adjust the *iso*:*anteiso* ratio and modulate membrane properties in response to the fluctuations in the environment (pH, temperature, pressure *etc*.) [36].

Another type of modification is called *cyclopropanation* and this is usually observed in bacteria entering into the stationary phase. Cyclopropanation is achieved by the conversion of pre-existing cis-UFAs to cyclic forms of the methylated fatty acids. This conversion is performed by a membrane-associated enzyme known as cyclopropane fatty acid synthase (CFA). CFA

transfers a methyl group from *S-adenosylmethionine* and retains the *cis*-configuration of the acyl chain [13]. Transcription of the *cfa* gene is controlled by the σ^s (sigma) transcription factor and activated as the cells enter stationary phase [13]. CFA interacts with anionic lipids in the membrane and thus surface charge is proposed to be important for the upregulation of *cfa* transcription and the increase in CL content during stationary phase seem to support this phenomenon [37-39]. Since there is no mechanism for reversing cyclopropanation, their content is diluted with newly synthesized cis-UFAs when cells re-enter logarithmic growth. The advantage of having cyclopropanated species arises from the high stability of the cyclopropane bond compared to SFAs and UFAs. Some pathogenic *E. coli* strains having higher levels of cyclopropanated fatty acids, were observed to be more resistant to acid stress, antibiotics and temperature shifts [13]. All these adaptations minimize the energy required for the maintenance of the electrochemical gradient and thus optimize all energy-dependent reactions in the bacterial metabolism [13].

The complexity of a biological membrane does not originate only from fatty acid variations but also from the diversity in the headgroups and lipid backbones.

2.2.2. Headgroup diversity

In bacteria, phosphatidic acid is the main precursor of all the *glycero*phospholipids (containing *glycerol* as the backbone) and slight modifications on the headgroup could generate a diverse collection of phospholipids with different physicochemical properties. The most important feature is the surface charge brought by the lipid headgroups. A diverse collection of lipid headgroups, is generated by bacteria to balance an optimum surface charge in the membrane [24, 40]. Maintenance of an optimum surface charge is important, since many enzymatic reactions are regulated by electrostatic interactions [13, 24]. Some membrane-associated enzymes, like PssA, are regulated by surface charge. For PssA, the dilution of negativity in the surface causes the inactivation of the enzyme and thus *zwitterionic* (+,-) PE levels decrease, leading to increased *anionic* lipid levels. Increased anionic lipid content contributes to the negative surface charge and reattracts PssA to the membrane thus PE levels again increase [41].

2.2.3. Backbone diversity

Some bacteria use alternative backbones to glycerol-3-phosphate when phosphate levels are low in growth medium. The common substitution is the replacement of glycerol backbone with amino acids such as ornithine (most common), lysine, glycine, glutamine and serine-glycine [40].

α-proteobacteria such as *Rhodobacter sphaeroides* and *Sinorhizobium meliloti* can replace the majority of their phospholipids with *ornithine* lipids and also have two non-phosphorous lipids: *betaine* lipids and *sulfolipids*. In cyanobacterial membranes, adaptation to phosphate-limiting conditions differs from other bacteria and resembles more to the adaptation observed for plants [42]. The economy for phosphate is provided by the phosphate retrenchment in the headgroups not in the backbone. *Glycolipids* (GlcDAG and GlcGlcDAG) and *galactolipids* (GalDAG and GalGalDAG) are the major constituents of cyanobacterial membranes and a sulfolipid, sulfoquinovosyl diacylglycerol (SQDG) is also present. SQDG is an anionic lipid like PG and its uncommon sulfoheadgroup makes this lipid chemically stable.

The second common backbone type observed in lipids is the *sphingoid* backbone. A sphingoid backbone is a long aliphatic amino alcohol N-linked to a fatty acid and O-linked to a charged group (ethanolamine, serine or choline). *Sphingolipids* are a class of lipids that contain sphingoid backbone and are classified as mechanically stable and chemically resistant lipids. They render bacterial cells to be more tolerant against oxidative stress and heat shock [40]. In *eukaryotic* cells, sphingolipids form microdomains in the membrane and these sphingolipid islets play significant roles in signal transduction and cell recognition [40].

2.2.4. Lipid/protein ratio

An important physicochemical property of a biological membrane is *membrane fluidity*. This property could be defined as the motional freedom of a solute molecule inside a lipid membrane. Besides, modifying fatty acids and headgroups, fluidity can be adjusted by changing the lipid/protein ratio. Proteins are considered to be more rigid structures than lipid molecules. Increasing the protein content therefore leads to more rigid membranes with low permeability and mechanically less stable membranes. In contrast, increasing lipid content will decrease the order and form a flexible, and mechanically stable membrane with high permeability. However, increasing the content of lipids with rigid structures, such as *cholesterol*, is an exception to this acceptance in *eukaryotes*. Higher cholesterol content in eukaryotic membranes is known to lower fluidity and thus create a rigid membrane with lower permeability to solutes. However, in *eukaryotes* membrane fluidity is regulated by another ratio: cholesterol/phospholipid ratio [43].

2.2.5. Bilayer-prone/Non-bilayer prone ratio

Attractive and repulsive forces between the headgroups and fatty acids of two lipids with different shapes usually create a lateral pressure in the mem-

brane. In order to understand this, different shapes of lipids and types of the curvature created need to be understood.

Membrane phospholipids usually have an overall cylindrical shape if the cross-sectional area of the headgroup is similar to the cross-sectional area of the acyl chains. Such lipids are called *bilayer prone* lipids and they aggregate to form a liquid-crystalline lamellar (L_α) phase, a phase that is usually seen in biological membranes (bilayer shape, zero curvature) (**Figure 4**). If the cross-sectional areas of headgroup and acyl chains are different from each other, these lipids are referred as *non-bilayer prone* lipids. There are two types of non-bilayer prone lipids in biological membranes. The first type has a larger headgroup compared to its acyl chains and forms positive curvature [21, 44]. Lipids with positive curvature form micelles in water and tend to have normal hexagonal H_I phase [44]. The second type of *non-bilayer prone* lipids has relatively small headgroups compared to their acyl chains (**Figure 4**). These lipids generate negative curvature and form an inverted conical molecular shape. They are capable of forming reverse hexagonal (H_{II}) phase in the bilayer [44]. Adjusting the bilayer prone/non-bilayer prone ratio is extremely important since the overall contribution of their molecular forces affects the mean curvature of biological membranes. Membrane curvature and its importance for cellular processes will be discussed later in this thesis.

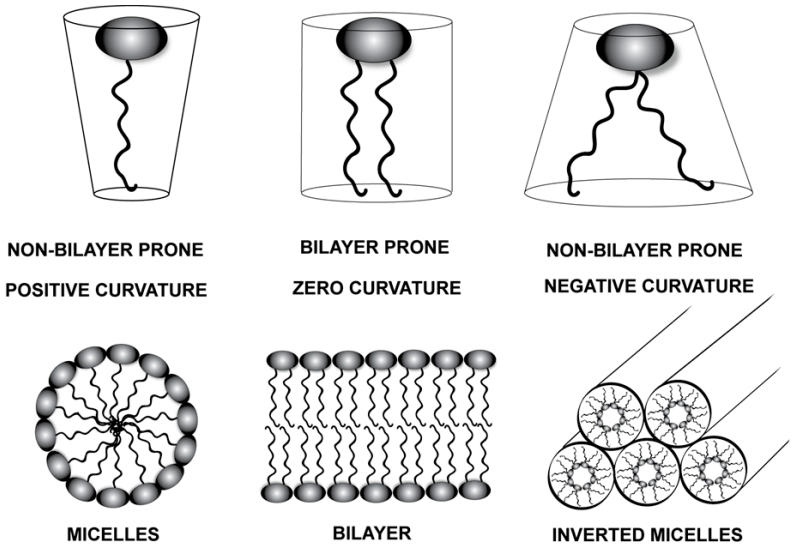

Figure 4. Shapes, types of curvature and self-assembly of lipids. Lipids with positive and negative curvature are grouped as non-bilayer prone lipids and tend to induce a curvature when aligned. Non-bilayer prone lipids with positive curvatures tend to form micelles and with negative curvature they form inverted micelles. However, bilayer-prone lipids do not tend to induce curvature, thus form a bilayer when aligned. Adapted from ref. [21].

2.3. Membrane Proteins

20-30% of all predicted genes encode membrane proteins (mostly helix-bundle) thus they constitute a major fraction of the protein universe [45]. Two distinct structures are usually seen: α-helix bundle and β-barrel. These two architectural structures formed by the intra-molecular hydrogen bonding of amino acid residues are buried deeply in a complex lipid environment. The types of interactions of membrane proteins with a lipid bilayer are vast and could have significant consequences.

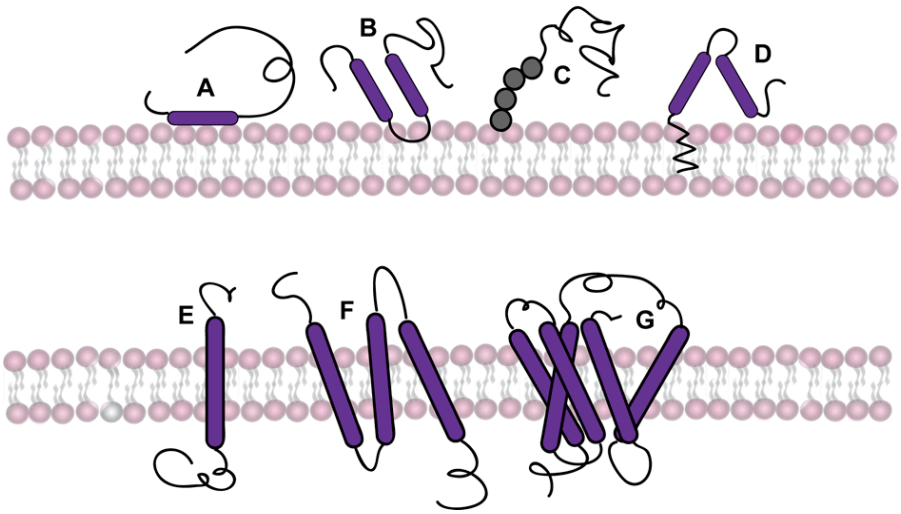

Figure 5 Peripheral and integral membrane proteins. Different types of interactions observed for different membrane proteins. Peripheral membrane proteins are represented above and integral proteins are given below. A. Monotopic membrane protein interacting via its amphipathic helix (MGS type or ALPS motif type); B. Monotopic membrane proteins with a hydrophobic patch residing in a loop; C. GPI-anchored membrane proteins as an exception to peripheral proteins but usually have purification properties similar to integral proteins; D. Lipid anchored membrane proteins are also considered as an exception like GPI-anchored proteins; E. Bitopic membrane proteins; F. Polytopic membrane proteins; G. Multimeric assemblies of membrane proteins (channels or transporters).

2.3.1. Peripheral (membrane-associated) proteins

During purification, some membrane proteins can be isolated using mild conditions (salts, cholates or HCO_3^-). These proteins usually have no trans-membrane domains and have higher content of α-helix bundles in their architecture [45, 46]. Although they do not have transmembrane domains, they

adhere to the membrane temporarily and their attachment can be in two ways: First, the membrane protein penetrates itself into the bilayer via ionic and hydrophobic interactions with the lipid headgroups. Second, the protein can have lipid (a covalently attached fatty acid such as palmitate or myristate) or glycolipid anchors as was observed for GPI-anchored proteins [47]. Although, proteins having the second type of attachment way are usually classified under peripheral proteins, their purification properties are similar to those of integral proteins. For this reason, they are considered as an exception to the definition of a peripheral membrane protein.

A subclass of peripheral proteins, *monotopic* membrane proteins only interact with the single leaflet of the bilayer and do not span the bilayer [48, 49]. Interaction can occur via hydrophobic patch on the protein surface as seen for prostaglandin synthetase [50, 51], or via an uncleavable signal sequence in their N-terminus as their anchor to the membrane such as cytochrome P450 enzymes [52].

The protein can have two types of amphipathic helices in their N-terminus: a classic amphipathic helix with alternating hydrophobic and positively charged amino acid residues [53] or an Amphipathic Lipid Packing Sensor (ALPS) motif. An ALPS motif has hydrophobic residues but it is highly enriched with polar residues (mostly serine and threonine) [54]. In contrast to a classic amphipathic motif interacting anionic lipids with their positively charged headgroups (MGS), an ALPS motif interacts with the membrane with its hydrophobic residues. The common features between two different motifs are their abilities to form α-helices when they face the bilayer. ALPS helices release the membrane via the binding weakness brought by its polar groups and classic amphipathic helices achieve this via their hydrophobic groups [53, 54]. These groups cause monotopic membrane proteins to be described as more loosely bound membrane proteins.

Although monotopic proteins are expected to be solubilised better and released from the membrane in a much easier way compared to integral membrane proteins, only a small number of monotopic protein structures are available in PDB. The reason for the low number of structures could be related to their flexible structures, which preclude them from forming protein-protein contacts during crystallization studies.

2.3.2. Integral membrane proteins

The most common type of membrane proteins are *integral* proteins spanning the bilayer once (*Bitopic*) or more than once (*Polytopic*) [48] (**Figure 5**). Bitopic proteins can be classified as type I or type II with respect to their N-terminus residing outside or inside the cytoplasm, respectively. Most mem-

brane proteins have more than one transmembrane (TM) segment connected with loops and they are considered as type III integral membrane proteins.

The topology of an integral membrane protein, is encoded by the primary amino acid sequence and then decoded by the translocon. Stability of the topology is maintained by interactions with membrane lipids. These interactions are hydrogen bonding, hydrophobic and ionic interactions. Since membrane proteins contain hydrophobic portions, the need for hydrogen bonding among polar amino acid residues is fulfilled by intermolecular hydrogen bonding (α-helical bundles or β-barrels). Ionic interactions are mostly take place between positively (Lys, Arg and His) or negatively (Asp and Glu) charged residues of proteins and lipid headgroups. A role for anionic lipids during the insertion and arrangements of TM segments of some membrane proteins such like leader peptidase (Lep), phenylalanine permease (PheP), gama aminobutyric acid permease (GabP), lactose peremease Y transporter (LacY) and potassium channel protein KcsA, has been reported [55]. Besides the stabilizing effect of the lipid headgroups, hydrophobic portions of TM segments are stabilized by the fatty acid moieties of membrane lipids.

In order to understand the roles of membrane lipids in shaping the structure and topology of a membrane protein, types of interactions taking place at the protein-lipid interface need to be understood. Therefore, a description of possible positions of lipids observed in some x-ray structures, is given in the next section of this thesis. The two interconnected phenomena, *membrane curvature* and *vesiculation* are also discussed with respect to their relevance for membrane protein-lipid interactions.

3. Lipid-protein Interactions

3.1. Types of Interactions

Lipids are often described by their proximity to the membrane proteins. Depending upon the position where these lipids are found they are termed differently and the type of interaction differs with the orientation of lipids with respect to the mid-plane of the bilayer. According to their proximity, lipids can be classified as 1st shell lipids, 2nd shell lipids and bulk lipids. A membrane protein has a significant effect on the properties of 1st shell lipids and indeed restricts the motional freedom of lipids compared to 2nd shell lipids and bulk lipids. Therefore, the properties of bulk lipids are not significantly affected by the presence of a membrane protein unlike 1st shell lipids [8].

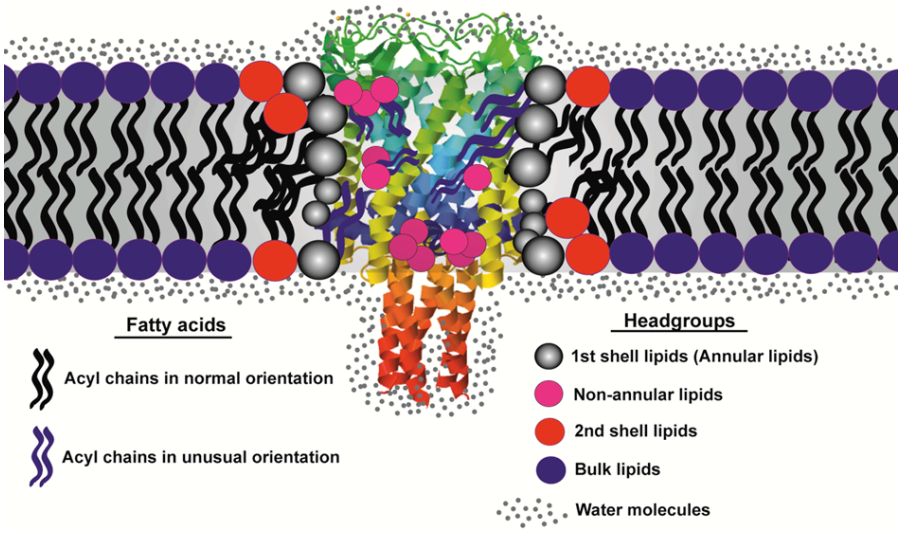

Figure 6. Lipid-shells around a membrane protein. The structure is represented is the Mechanosensitive Channel of Large Conductance (MscL) (PDB code: 2OAR).

3.1.1. 1st shell lipids (Annular lipids)

The 1st-lipid shell is considered as a *lipid annulus* since it resembles a ring around the membrane protein (**Figure 6**). The lipids forming this annulus are termed as annular lipids and have much slower exchange rates with bulk lipids (1-2 x10^{7}s^{-1} at 37°C) [8, 56]. The time of an annular lipid to stay at-

tached to a membrane protein depends on the affinity of the relevant amino acid residue for the lipid molecule of interest. If this interaction between the membrane protein and the lipid molecule is strong, then this lipid molecule has slow off-rate compared to bulk lipids and usually this interaction point is considered as a *hot spot* for lipid binding. The total interaction between all lipids and amino acid residues of a membrane protein is usually given by the *total interaction energy*, which represents the sum of all interactions (van der Waals interactions, hydrogen bonding, ionic interactions etc.). However, total interaction energies fluctuate greatly since annular lipids are not fixed at their positions and they move freely in and out of the membrane protein annulus. A slight preference for a specific type of lipid could be observed when a membrane protein is purified even if the membranes contain a low concentration of that specific type of lipid.

Annular lipids can have regulative effects on the functions of membrane proteins. The most common examples of function-regulating annular lipids are found to exist in the structures of potassium channels (Human Kir2.2 and bacterial KcsA) and the large-conductance mechanosensitive channel (MscL) [57, 58]. MscL from *Mycobacterium tuberculosis* is a pentameric osmoregulative protein composed of two transmembrane components, TM1 and TM2, with a cytoplasmic α-helix (**Figure 6**) [56, 59]. In MscL, a cluster of three positively charged residues Arg98, Lys99 and Lys100 form a *hot spot* where anionic lipids are retained on the protein surface and released upon deprotonation [60-62]. A RKKEE motif found in the C-terminus of MscL is highly conserved among the prokaryotic and eukaryotic mechanosensitive (MS) channels [59]. These lipid-binding motifs are usually found in the linear sequence as indicated for MscL but it is not obligative for the formation of a hot spot for lipid binding. The other example Kir2.2 partially binds two anionic lipids but the lipid binding takes place where Lys188, Lys189 and Arg109 form a pocket for anionic lipids like PtdnIns(4,5)P2 to bind [8, 62]. There is no clear sequence rule for anionic lipid binding but motifs containing **RK**, **RR**, **KR** or **KK** are most likely to be hot spots for lipid binding.

3.1.2. 1st shell lipids (*Interfacial Lipids*)

The maintenance of membrane potential and proton motive force is crucial for the cells to perform some important processes such as ATP synthesis, ion homeostasis, molecular transport and trafficking etc. [63]. Membrane proteins however have indented surfaces consisting of crevices and gaps. *Interfacial lipids* act as molecular glue that seals any aperture that could depolarize the membrane potential.

Most of the lipids structurally resolved frequently reside at the contact sides between the monomeric units of oligomeric assemblies. The first example of this type of lipid was the haloarcheal glycolipid S-TGA-1 (3-HSO_3-Galpβ1-6Manpα1-2Glcpα-1-archaeol) detected in the 2.9 Å x-ray structure of bacteriorhodopsin [64]. It was concluded that this glycolipid acts as a molecular-glue that interlocks two unmatched monomers. Interfacial lipids are also observed in the structures of ion channels, proton pumps or receptors [64, 65].

3.1.3. Non-annular Lipids

Another class of lipids found in the membrane protein structures are *non-annular* lipids. These lipids are usually buried deep within the clefts and crevices of membrane proteins. They are similarly attached to the membrane protein surface as annular lipids but they are somehow trapped in small pockets/crevices formed by amino acid residues in unusual positions (headgroup below the membrane plane and/or nonperpendicular to the bilayer) and have much lower off-rates compared to *annular lipids* [8, 58, 64]. Some non-annular lipids have major roles in the regulation of membrane protein function by contributing to their folding and self-assembly in the membrane [64]. One of the most recognized examples is phosphatidylinositol (PI), which is observed in the structure of the cytochrome bc_1 complex. PI resides in an interhelical (an unusual) position where side chains of cytochrome bc_1 stabilize the headgroup of PI through the formation of several hydrogen bonds with the inositol headgroup. Lipid binding dissipates the torsion forces generated by the fast movement of the extrinsic domain of the Rieske protein. Hence PI acts as a regulator molecule for the cytochrome bc_1 complex and could also be important for the self-assembly of the complex for it is located at a position where four TM subunits of the monomer come in contact with each other [64].

Combinatorial motifs of positively charged and polar amino acid residues (KT, KW, KY, RS, RW, RY, RN, HS, HW and HY) are reported to stabilize the lipids with phosphodiester groups (excluding PC). For cardiolipin, three residue motifs such as KKY, RKY and HRN have been suggested [64]. Usually anionic lipids are observed in the structures of membrane-protein complexes but other types of lipids have also been noted. In cytochrome c oxidase from *Rhodobacter sphareoides*, six phosphatidylethanolamines (PE) were identified [66, 67].

The majority of lipids identified in x-ray structures reside on the electronegative side of the membrane (n-side, mitochondrial matrix & cytoplasmic side of the plasma membrane). However, lipid headgroup stabilization at the electropositive side (p-side) might occur less frequently [64].

3.1.4. Bulk Lipids

Bulk lipids can also affect membrane protein function as they contribute to the macroscopic properties of the membrane. These properties include viscosity (fluidity), internal pressure (lateral stress) and spontaneous membrane curvature (curvature elasticity) [2].

Membrane curvature influences a diverse variety of processes such as membrane fussion and fission, molecular transport, recruitment of proteins into the cytoplasmic surface, regulation of the activities of membrane proteins, molecular trafficking etc. [68]. Biological membranes often contain lipids that adopt the hexagonal (H_{II}) or non-bilayer phase. By modulating the proportion of bilayer and non-bilayer lipids, it is possible to modulate membrane curvature.

3.2. Membrane Curvature

Membrane curvature defines the ability of a membrane to curve its midplane towards the interior or exterior of the bilayer itself. This movement requires some work. According to calculations based on the Helfrich model [69], to completely bend a flat bilayer and form a mole of closed spherical bilayer requires 200-250 kcal of energy [70, 71]. This energy is created in the bilayer by the free-energy release arising from the spatial arrangements of lipid headgroups and chains.

Many lipids mostly contain a glycerol backbone (Glycerolipids) and just below the lipid headgroup region, an attractive force $F\gamma$ arises from the unfavourable interaction of acyl chains with water molecules (hydrophobic effect) (**Figure 7**). $F\gamma$ is opposed by two different repulsive forces in the bilayer. Acyl chains of lipids are hydrophobic and prefer not to be exposed to the water phase so they pack tightly with each other. This creates a negative lateral pressure in the membrane. As they pack so tightly, thermal motions of acyl chains can create a repulsive force (F_c) that expands the membrane. The other repulsive force (F_h) arises in the headgroup region where steric, hydrational and electrostatic features of different lipid headgroups are in conflict with each other. Although F_h is described as a repulsive force, it might contain some attractive forces such like hydrogen bonding with polar headgroups of other lipids.

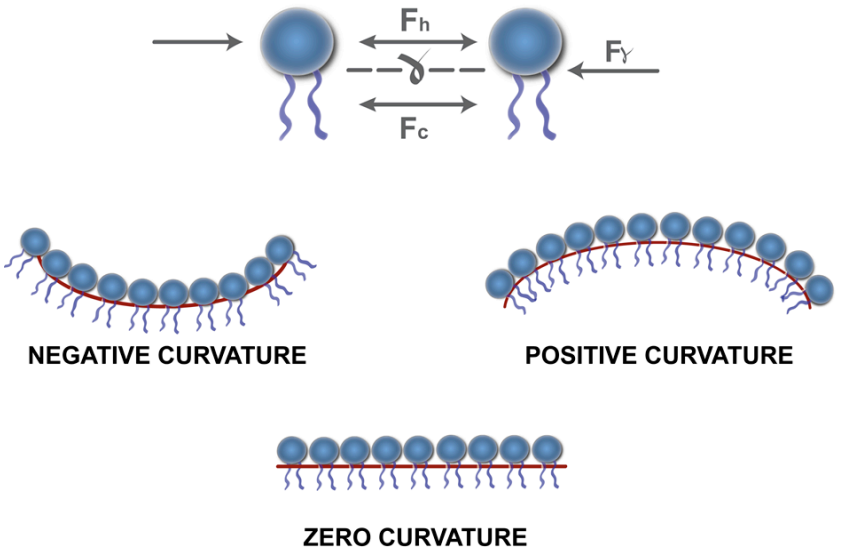

Figure 7. Forces acting on lipids at the membrane interface promote different curvatures. The shape of a lipid molecule is also affected by these forces; $F_c > F_h+F_\gamma$ or $F_c < F_h+F_\gamma$ forms a non-bilayer shape lipid (see **Figure 4**), which creates negative or positive curvatures, respectively. Biological membranes have also bilayer-prone lipids, which usually have zero curvature where $F_c = F_h+F_\gamma$. Adapted from ref. [2].

To create a flat membrane, F_c and F_h should be in balance with Fγ. If the lateral pressure in the chain region becomes greater than that between the headgroups ($F_c > F_h + F\gamma$), the bilayer will bend and curl towards the aqueous region (*negative curvature*). On the other hand, *positive curvature* is created when lateral pressure in the headgroup region surmounts the forces acting in the acyl chains ($F_c < F_h + F\gamma$). In a flat bilayer (*zero curvature*), two monolayers will counteract each other and either monolayer could not be curved. This is called as *curvature frustration* and has been suggested to be important for the proper functioning of the bilayer [2]. Some factors impair the critical balance of forces acting in the bilayer. The most acknowledged mechanisms are: spontaneous curvature formation by lipid asymmetry, protein scaffolding, insertion of wedged-shape proteins and local crowding (**Figure 8**) [70]. In this PhD thesis, the first two mechanisms will be discussed since they are significant to understand the vesiculation by MGS of *A. laidlawii* (Papers I and II).

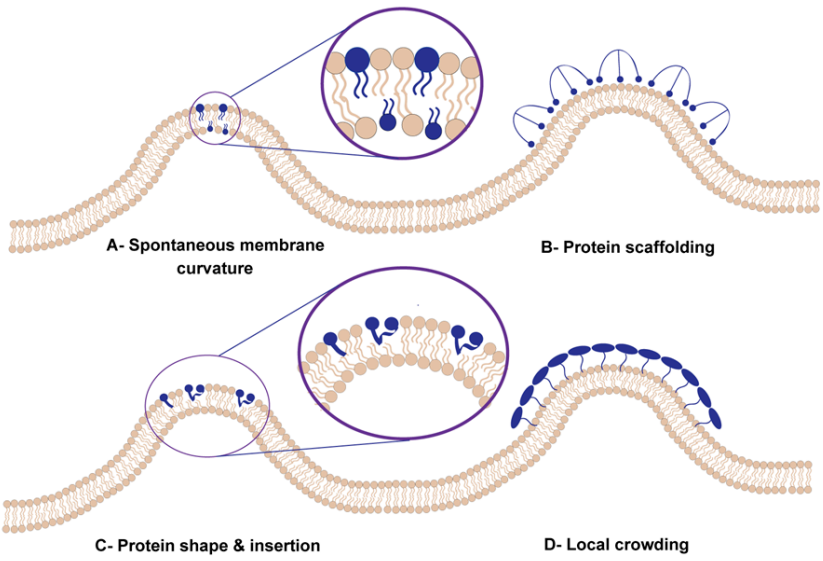

Figure 8. Different mechanisms of membrane curvature formation. A. Spontaneous membrane curvature formation by the lipid asymmetry; B. Protein scaffolding (BAR proteins); C. Insertion of proteins with wedge shapes can create an asymmetry in a bilayer; D. Accumulative binding/insertion of a protein to specific lipid domains can deform the bilayer. Adapted from ref. [70].

3.2.1. Modulating membrane curvature by lipid asymmetry

Lipid asymmetry is an important factor for the generation of membrane curvature. A simple description of lipid asymmetry could be the unequality between the surface area ratios of two monolayers [72]. There are two ways to generate membrane asymmetry in a flat bilayer. The first way is to change the lipid composition of monolayers so they become different in terms of either total amount of lipid molecules, or diversifying lipid species (modifications of headgroups or fatty acids), or both [71]. Some membrane proteins could influence the properties of fatty acids/headgroups through binding to specific lipids and thus create an imbalance in the bilayer (Paper II). The molecular mechanism for the regulation of fatty acid/headgroup modifications is not yet clear. However, some suggestions indicate a controlling mechanism dependent upon local surface charge fluctuations [73]. Since all lipid-synthesizing machinery is located in the bilayer, it is logical to think that any decrease or increase in the surface charge within the bilayer could influence the enzymes responsible for lipid synthesis.

Phosphatidylserine synthase (PssA) is an enzyme responsible for the synthesis of a non-bilayer prone lipid, PE, in *E. coli*. Activation of PssA depends

on its selective interaction with PG and CL through the positively charged residues concentrated at the N- and C- termini of the protein [41, 74]. This specific interaction with PG and CL leaves a non-bilayer prone lipid (PE) alone, which affects the curvature properties of the bilayer. The PssA activation mechanism is simply explained by ionic interactions with the bilayer interface [74, 75], but the effects of the PssA protein on the fatty acid modeling and membrane properties have not been studied in detail. Preferential binding of lipids with specific acyl chain length can also change the symmetry in a bilayer, which induces membrane curvature.

Paper I and II demonstrate the selective binding of MGS and its role in inducing fatty acid remodeling in *E. coli* membranes. Preferential binding to PG and CL on one membrane leaflet leaves a non-bilayer lipid on the other leaflet, which can create membrane asymmetry in *E. coli* membranes. Furthermore, changes in fatty acids especially of those of PE can induce an asymmetry in the membrane and we believe these factors all contribute to the formation of intracellular vesicles in *E. coli*.

3.2.2. Modulating membrane curvature by protein scaffolds

The shape of the scaffold protein as seen in BAR proteins [68, 70] forces the membrane to curl and membrane curvature is generated. BAR proteins have a conserved structural pattern consisting of three helix coiled-coiled motifs, which form curved homo- or heterodimers. These oligomeric structures give BAR proteins a characteristic “banana shape” and force the whole membrane to follow their curved structure [76]. It should be noted that the shape of the protein is not the only factor for the generation of curvature. Protein crowding on the membrane surface may influence the local surface density of the proteins. The occurrence of protein islets disrupts the homogeneous distribution of both lipids and membrane proteins and leads to an asymmetry in the membrane causing curved protrusions.

Besides its scaffolding shape, a membrane protein could bend the membrane by the insertion of its amphipathic helix. This disturbs the homogeneous distribution of lipids by withdrawing some specific lipids interacting with the amphipathic helix (Paper I). MGS from *A.laidlawii* has an amphipathic helix with 9 positively charged residues interacting particularly with anionic lipids (PG and CL). BAR proteins also have 12 positively charged residues on their concave surface allowing the protein to interact with anionic lipids, e.g. PtdIns(4,5)P_2 [76]. Withdrawal of minor species such as anionic lipids from the membrane lipid pool might disturb locally the force-balance acting upon the lipid bilayer.

3.3. Intracellular Vesicles

Although *E. coli* do not normally generate intracellular membranes, the formation of additional invaginations from the inner membrane, tubules, sacks or even vesicles have been reported when native or foreign membrane proteins are overexpressed [77-83]. Although they are different proteins at structural and functional levels, they have some common features. Here these similarities will be described and an attempt to identify a common pattern amongst these vesiculating proteins will be made.

3.3.1. Vesiculation by MGS

Monoglucosyldiacylglycerol synthase (MGS; 2.4.1.157) is a GT-B type (GT-B fold) glycosyltransferase (**Figure 9**) responsible for the formation of monoglucosyldiacylglycerol (GlcDAG) by a glucosyl group transfer from UDP-glucose to diacylglycerol (DAG) in A. *laidlawii* [48, 77, 84, 85].

MGS causes intracellular vesicles when overexpressed in *E. coli* cells [77]. When vesicles were analyzed for their lipid distribution, a minor enrichment of PG and CL lipid was observed [77]. Additionally, the analysis of protein distribution in isolated vesicles indicated that 90% of all proteins found in the vesicles were MGS molecules [86]. These MGS molecules are most likely located on the outer surface of the vesicles. The remaining 10% of proteins constitute 17 different proteins of the cytoplasm, inner and outer membranes [86]. The random distribution of other proteins and their unchanged ratios relative to control cells suggests that they do not influence the vesiculation process.

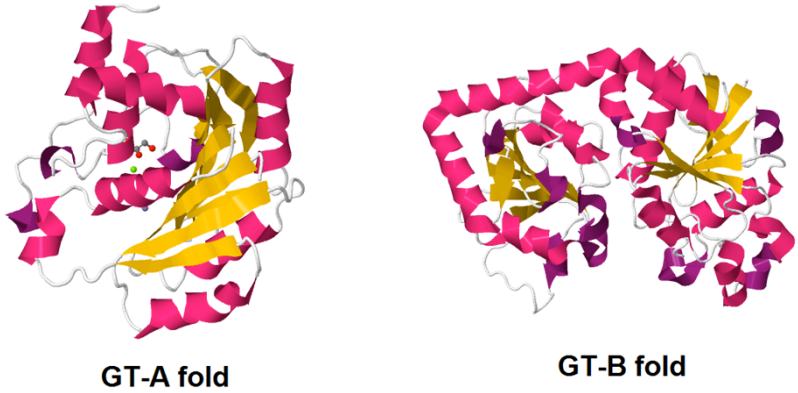

Figure 9. GT-A and GT-B type of glycosyltransferases. Two main subclasses of glycosyltransferases, GT-A and GT-B are represented by the nucleotide-disphosphosugartransferase (spsA) from *Bacillus Subtilis* (PDB code: 1QGQ) and β-glucosyltransferase from bacteriophage T4 (PDB code: 1QKJ), respectively.

The molecular mechanism or reason/s behind the vesiculation process has remained unclear so far. However, a simple mechanism was proposed [77] that pointed to a possible connection between vesiculation and anionic lipid binding by MGS. Paper I and II, focus on the discriminative withdrawal of anionic lipids from the membrane by MGS binding and the events leading to the release of vesicles from the inner membrane.

As a monotopic membrane protein, the membrane binding properties of MGS were observed to be dependent on the hydrophobic and ionic interactions with anionic lipids *in vitro* [84]. An amphipathic helix of MGS at the position 65-87 (65-SL**K**GF**R**LVLFV**KR**YV**RK**M**RK**L**K**L-87) with 9 positively charged residues are responsible for the specific interaction with anionic lipids *in vitro* [53]. In this PhD thesis, this discriminative binding preference of MGS was validated *in situ* for the first time (Paper I). Furthermore, lipid amounts per cell were doubled and were proportional to anionic lipid production. In connection to stimulated lipid synthesis, MGS levels were upregulated 2.8 fold.

3.3.2. Vesiculation by other membrane proteins

Intracellular vesicle, tubule or sack formation have also been observed with other membrane proteins [79, 81, 87]. There are some similarities between MGS and these proteins. Most vesiculating-proteins have a hydrophobic segment (usually found at their N-terminus) enabling them to be attached to the membrane surface. These hydrophobic segments contain or are surrounded with positively charged residues providing a larger contact surface with the phosphate headgroups of the bilayer phospholipids [53, 79, 88-90]. This provides a stronger attachment to the membrane through both hydrophobic and electrostatic interactions. MGS has a strong discrimination against anionic species in the membrane (Paper I) and other vesiculating-proteins apparently follow the same trend [79, 83, 89-91]. LpxB is an exception as it was found to be associated mostly with PE in the membrane with a lipid/protein ratio of 1:1.6 [78]. The third most striking similarity between MGS and other vesiculating-proteins are their great ability to double the amounts of phospholipids. However, there is one difference: MGS leads to an increased lipid/protein ratio, whereas others keep lipid/protein ratios essentially constant [79, 81, 82, 87, 92].

All lipid synthesizing machinery of a cell (including PlsB, the first enzyme in phosholipid synthesis) resides inside the lipid bilayer [40]. Due to the neutralization of negative charges upon binding, charge fluctuations occur in the membrane and are sensed by the individual components of the lipid synthesizing machinery. The regulation mechanism of all the enzymes in the

phospholipid synthesis pathway remains unknown. Considering the importance of anionic lipids for the regulation of some membrane proteins such as PssA [93-95], PSD [96] and CFA [38] , it is not so difficult to conclude that any change in the surface charge within the bilayer would be sensed and thus activate all the enzymes within the membrane.

To my knowledge, there is no hypothesis in the literature that connects the doubled phospholipid amount with discriminative anionic lipid binding. In Paper I, a correlation between upregulated phospholipid synthesis and selective anionic lipid withdrawal from the membrane was noted. In addition, the activation of the lipid synthesis pathway was revealed (Paper I). CFA deactivation during MGS overexpression was a clear indication of a strong communicative pathway between all the enzymes associated with the membrane (Paper II). Since CFA [38] and MGS (Paper I) are both known to interact with anionic lipids, their communication depends strongly on the negative charge levels brought by the anionic lipids acting as messenger molecules in the membrane. A similar communication could exist for other enzymes in the lipid synthesis pathway.

4. Methodology

4.1. Model Membrane Systems

Since a biological membrane is a fluctuating dynamic environment with numerous membrane proteins embedded, observation of a single molecular interaction *in situ* is impossible with existing biochemical and biophysical methods [97]. Therefore model systems mimicking the actual membranes have been developed. Three major classes of membrane mimicking systems that were used in this PhD thesis will be described in detail: *micelles* (Paper III), *bicelles* (Paper I) and *vesicles* (Paper IV).

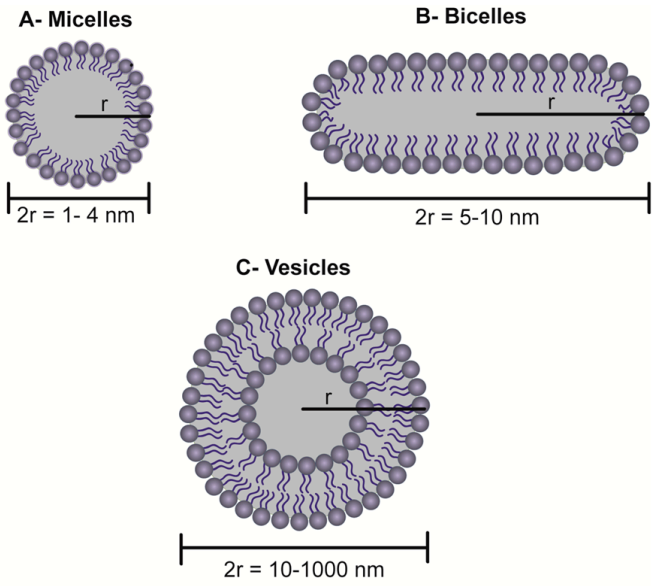

Figure 10. Model membrane systems. A. Micelles have small sizes and thus form high curvatures, which distort peptide/protein binding onto the micelle surface; B. Bicelles have a flat surface in the midplane and have less curvature compared to micelles, hence they are considered as a better membrane-mimicking system; C. Vesicles are considered to be a moderate model system since they have a large surface area and lower membrane curvature,which better mimicks a biological membrane.

4.1.1. Micelles

The simplest way to mimic a biological membrane is to use lipids dissolved in an aqueous environment (micelles). Since lipid molecules are hydrophobic, they tend to aggregate in water exposing polar headgroups and orienting

the hydrophobic acyl chains towards the micellar core. Aggregation occurs spontaneously when a certain concentration of the lipid is dispersed in a polar solvent (water). The concentration of a surfactant molecule (lipids or detergents) at which micelles start to be formed is called the *critical micelle concentration* (CMC) [97, 98]. The CMC is an important characteristic for lipids and detergents. For every surfactant molecule, the CMC is a fixed number at a certain temperature and pressure and could differentiate when ionic molecules are present [98]. For a non-ionic detergent, n-Dodecyl-β-D-Maltoside (DDM), the CMC is 0.17 mM in H_2O but changes into 0.12 mM in the presence of 0.2 M NaCl (www.affymetrix.com).

Lipid-detergent micelles are good membrane mimicking models and more closely resemble a natural lipid bilayer where the membrane protein of interest is embedded *in vivo*. The biggest advantage of using micelles is their relatively small size [99], high curvature and their defined composition of lipids (**Figure 10**). Sometimes the high curvature of micelles could be considered as a disadvantage if the activity of the membrane protein is decreased with high curvatures. In Paper III, various lipid-detergent mixed micelles are used in the MgdA activity assays. The acceptor substrate (dioleoylglycerol, DOG for MgdA) could easily be incorporated into the micelles, which made it possible to observe activity changes in a defined lipid environment. The sizes and shapes of the micelles could vary with different detergents, lipids with different alkyl chains and buffer systems [99].

4.1.2. Bicelles

Bicelles or *bilayered-micelles* are also used as a model system in biochemistry and biophysics. Bicelles are a mixture of long (Dimyristoyl phosphatidylcholine, DMPC) and short chain lipids (Dihexanoyl phosphatidylcholine, DHPC) prepared in aqueous solvents [100, 101]. In aqueous environment, long chain lipids assemble into lamellar bilayer sheets thus forming the centerpiece and short chain lipids prefer to be in the micellar phase forming the rims of the bicelle (**Figure 10**) [102]. Since they have a flat surface in their center, they are more close to a natural lipid bilayer and this feature eliminates the disadvantage of high curvature of micelles.

The morphology of a bicelle is described by the ratio of long chain lipid/short chain lipid (q-value) and temperature [103-105]. The size of the bicelle depends on its q-value and bicelles with large q-values ($q>2.3$) can align themselves within the magnetic field [97, 101, 106]. However, smaller bicelles with $q<0.5$ form disc-shapes and tumble fast enough so they are eligible for liquid-state NMR spectroscopy [102]. Other than the q-value, one must pay attention to the preparation method of the bicelles to have a defined size and shape. There are four main strategies for the preparation of

bicelles after mixing long chain lipids with short chain lipid: vortex-centrifugation cycles, heating-cooling cycles, sonification and freeze-thaw cycles. Freeze-thaw cycles create smaller bicelles than other three methods [106] so the bicelles used in Paper I were prepared by freeze-thaw cycles. Diffusion coefficient measurements revealed the presence of small fast-tumbling bicelles with 3.9-4.2 nm in size (q=0.5).

4.1.3. Vesicles

Vesicles could be classified as *natural* (intracellular membranes) or *artificial vesicles* (liposomes). Frequently, artificial vesicles are used in biochemical and biophysical studies since they have a more defined composition and size [107]. A liposome containing a single bilayer is said to be *unilamellar*. *Multilamellar liposomes* contain more than one bilayer and resemble onion structures [108]. Three groups of liposomes exist: Small Unilamellar Vesicles (SUV), Large Unilamellar Vesicles (LUV) and Giant Unilamellar Vesicles (GUV) with diameters of 10-50 nm, 100 nm-1 μm and 1-100 μm respectively [109, 110]. The sizes of the vesicles are inversely correlated to the local curvature. Some biological molecules (proteins, peptides etc.) are affected negatively by high curvature profiles, thus larger vesicles are better model systems to study their interactions with lipid molecules.

In Paper IV, two different types of LUVs were used to study the effect of lipid environment on CpxA autophosphorylation and phosphotransfer activities. First, synthetic phospholipids were used to prepare proteoliposomes. However, the CpxA activity was lower in synthetic phospholipid LUVs compared to LUVs prepared from original *E. coli* lipids. Due to the fine adjustment of average acyl chain length by bacterial lipid machinery, natural lipids with fatty acids of 16.5 C length had scored over synthetic ones. This again shows usage of natural lipids in the preparation of artificial vesicles (liposomes) could be a better way to test membrane protein activity (Paper IV). Although artificial liposomes are considered as a model system that is more close to a natural membrane, natural vesicles are a better choice since their formation is under a strict control in *E. coli*. The intracellular vesicles formed by MGS from *A. laidlawii* can also be considered as a model system to study membrane protein-lipid interactions *in vivo*. Membrane proteins can be co-expressed with the MGS protein and the extra membrane surface could be used to accommodate the membrane protein of interest or vesicles can be isolated from cells and further use in *in vitro* studies.

4.2. Approaches to Study Lipid-Protein Interactions

4.2.1. Lipid-Fishing Method

Membrane proteins are within a sea of lipids and some of these lipids stay tightly bound during the extensive purification process of a membrane protein [64, 65]. Usual purification methods used during structural studies such as gel filtration chromatography using high pressures or silicagel columns are harsh and delipidate membrane proteins. The lipid-fishing method (LFM) however is a milder technique and maintains tightly-bound lipids in the membrane protein structure (Paper I).

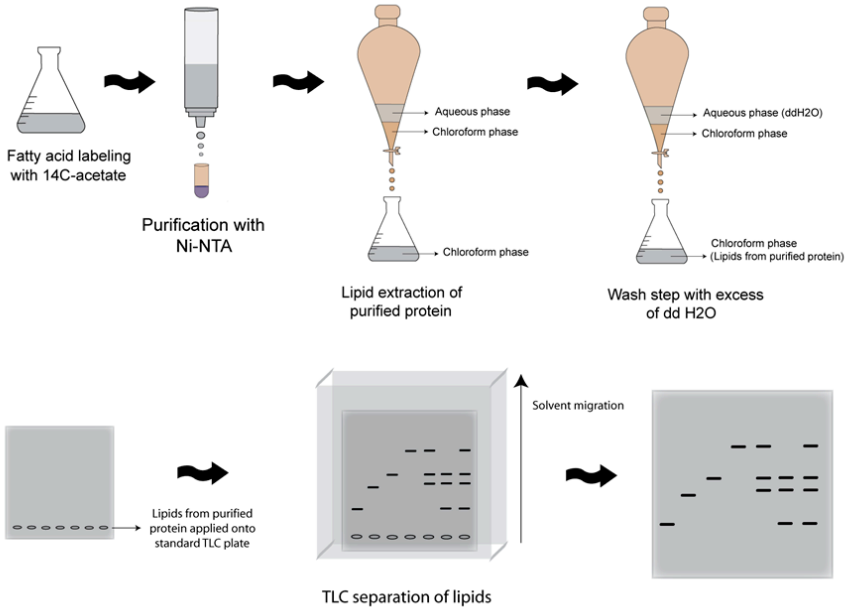

Figure 11. The lipid-fishing method (LFM). ^{14}C-labelled membranes are solubilised with DDM and purified by Ni-NTA chromatography under very mild conditions. After purification, lipids are reextracted and washed thoroughly with water. The extracted lipid mixture is separated by TLC and results are visualised by autoradiography.

The method is based on radioactively labeling all membrane lipids with ^{14}C-acetate. Acetate is incorporated into the acyl chains of lipids via lipid synthesizing machinery and proteins are solubilised using a very mild non-ionic detergent, dodecyl-maltoside (DDM) in this case (**Figure 11**). Since proteins to be purified contained 6-His tag at their N-terminus, affinity chromatography using Ni-NTA resins gave a very high purity (90% for MGS). However,

control experiments with empty plasmids had no signs of background protein binding. The tightly-bound lipids were then extracted using a standard Bligh-Dyer method [111]. The most difficult part of this method was to spot the lipids using thin layer chromatography (TLC) because DDM molecules that were co-extracted with the lipids resulted in a "wavy" TLC profile. In order to overcome this difficulty, DDM molecules were removed using liquid-liquid partition chromatography (LLPC). Since lipids are insoluble in water but DDM is soluble, chloroform phases were extensively washed until they become clear in colour and DDM-free. Chloroform phases were separated by TLC and visualized using autoradiography. Radioactive labeling enabled the detection of very low quantities of lipids (picogram scale) and a calibration was obtained by spotting a series of acetate label (1-^{14}C-acetate) between 25-3750 nCi before the exposure of samples with the phosphoimager screen for 20 hours. Using a calibrated autoradiography and some statistics about *E. coli* (http://ccdb.wishartlab.com/CCDB/), we were able to calculate lipid numbers associated with MGS (Paper I).

4.2.2. Phosphorous NMR (^{31}P-NMR)

Phosphorous NMR (^{31}P-NMR) is an analytical technique that analyzes the phosphorous (P) atom in biological compounds and provides information about the surroundings of the P atom [112]. The phosphorous atom is a *fermion*, which has a half integer spin (S= ½) and particles with ½ spin have permanent magnetic moments along their spin direction. This magnetic moment causes phosphorous atoms to be oriented in a single direction perpendicular to the magnetic field [105]. Any interaction between a phosphorous atom and another molecule causes a deviation from this orientation thus results in a chemical shift in the ^{31}P-NMR spectra.

Since all phospholipids in biological membranes contain phosphate headgroups [112], ^{31}P-NMR is widely used to monitor lipid-protein (lipid-peptide) interactions and could inform about bilayer packing of lipids, phase transitions (crystalline phase, gel phase etc.), lipid headgroup orientation/dynamics and elastic properties of lipid bilayers. In order to analyze lipid-protein interactions, a good fast tumbling membrane model has to be used. Bicelles have relatively smaller sizes, resemble more like natural bilayers with their flat regions, have distinct magnetic alignment properties and could be easily formed using a vast collection of lipids. All these features make them a popular choice in ^{31}P-NMR studies.

Usually commercial lipids are a common choice in bicelle preparations, however the details about growth conditions or strains from which the lipids are extracted are unknown. Since the fatty acid and headgroup compositions

differ with different strains and growth conditions [40, 93, 113], it is very hard to establish a very defined system. Also using lipids obtained from one source and trying to observe the interaction between these lipids and a protein from another source is not an ideal approach. In Paper I, lipids were extracted from the same source (BL21 AI cells) that MGS protein was obtained from and used during bicelle preparations. Since cells strictly regulate fatty acid and headgroup composition, this method makes bicelles a more ideal model system.

4.2.3. Fourier Transform Infrared Spectroscopy

Infrared spectroscopy focuses on the interactions of molecules with the electromagnetic radiation within the infrared region. Electromagnetic radiation can be described as the stream of particles called *photons* moving at the speed of light. Each photon contains a certain amount of energy given by the Bohr equation ($E=h\nu$; h is the Planck constant and ν is the frequency).

All biological molecules contain chemical bonds in their molecular structure. When a photon is absorbed, its energy causes each chemical bond to vibrate differently depending upon the energy amount given by the Bohr equation. Infrared spectroscopy exploits that molecules absorb specific frequencies that are characteristic of their structure. These absorptions are termed as *resonant frequencies*, in which the frequency of the absorbed radiation matches the transition energy of the bond or group that vibrates. This absorbed energy could be translated into an absorption spectrum where every single band in the spectrum correlates with the photon energy absorbed by a particular vibration. Each band in an infrared spectrum can be assigned to a particular vibration (Table 1).

Infrared spectroscopy deals with a certain region of the magnetic spectrum, the infrared region, which can be divided into three sub-groups:

a) Near-infrared region: 0.78-2.5 μM wavelength (12820-4000 cm^{-1})
b) Mid infrared region: 2.5-25 μM wavelength (4000-400 cm^{-1})
c) Far infrared region: 25-250 μM wavelength (400-40 cm^{-1}) [114]

Most vibrational energies of biological molecules fall within the mid-infrared region of the electromagnetic spectrum. Therefore mid-IR region was used in this PhD thesis (Paper I and II).

Table 1. Infrared band assignments for water, heavy water, lipids and proteins [114, 115].

		Band position / cm^{-1}	
Biomolecule	**Chemical bond**	**Mean value**	**Range**
H_2O	O-H stretching	3400	3800-3000
D_2O	O-D stretching	2600	2700-2500
Lipids	Antisymmetric CH_3 (choline) stretching	3038	3100-3000
Lipids	Antisymmetric CH_3 stretching	2956	2960-2950
Lipids	Antisymmetric CH_2 stretching	2920	2930-2910
Lipids	Symmetric CH_3 stretching	2870	2880-2860
Lipids	Symmetric CH_2 stretching	2850	2860-2840
Lipids	C=O stretching	1740	1780-1700
Lipids	Antisymmetric PO_2^- stretching	1228	1300-1200
Lipids	Symmetric PO_2^- stretching	1085	1100-900
Proteins	Amide I	1650	1700-1600
Proteins	Amide II	1540	1580-1510
Proteins	Amide III	1300	1400-1200

Fourier Transform Infrared spectroscopy (FT-IR) is a widely used technique to study membrane protein function and to characterize the properties of membrane proteins and lipids [116]. Since it deals with vibrational motions, any change that affects the vibration of a chemical bond (binding to lipids or aggregation of proteins) will be reflected in the spectrum (Paper II). The main difference between traditional infrared spectroscopy with FT-IR is the fast Fourier-transform (FFT) algorithm developed by Cooley and Tukey [117], which made it possible to analyze data faster and more accurately. This algorithm enables the transformation of energy amounts absorbed by the bonds into the wavenumber (cm^{-1}) in relation with their corresponding absorbance units (AU). Wavenumber is the reciprocal of the wavelength (cm) and have the advantage of being proportional to the amount of energy absorbed [114].

4.2.4. Cryo-transmission electron microscopy

Cryo-transmisson electron microscopy (Cryo-TEM) is a widely used technique for studying the intracellular membrane formation in bacteria [77, 87, 92, 118]. The method does not require chemical fixation, drying or staining of the sample. The sample to be analyzed only has to be vitrified in liquid ethane and kept at low temperatures during the experiment. This unique feature enables direct sample visualization.

In Paper II, intracellular vesicle formation was monitored in *E. coli* in a time dependent manner by cryo-TEM. A small volume of the sample (~1 μl) was placed onto a copper grid perforated with a polymer film (holey polymer film) and excess sample was removed by a filter paper, leaving a very fine thin film on the sample holder [119]. The formation of a very fine thin film is important since an electron beam does not penetrate into the sample deeper than 500 nm. The copper grid was rapidly dipped into liquid ethane at -183°C to vitrify the sample and rest of the experiment was performed at -183°C. Working at low temperatures is the most essential issue about cryo-TEM since samples might be disturbed at high temperatures and ice crystals could be formed without the sudden vitrification step [120].

An electron beam is projected on the polymer film that carries the sample to be analyzed. The electron beam is scattered by the particles (cells and vesicles inside the cytoplasm) in the sample. Image visualization is achieved by observing differences in the electron densities of vitrified cell membranes and solvent/buffer. Since a very limited area in the sample could be visualized with cryo-TEM, many micrographs have to be analyzed.

4.2.5. GFP-based promoter activity assay

The Cpx and σ^E are two major stress responses in *E. coli* [121, 122]. They are involved in the maintenance, adaptation and protection of the bacterial envelope in response to a variety of stress factors and are highly conserved among gram-negative bacteria especially pathogenic species [123]. The σ^E envelope stress response maintains outer membrane homeostasis and its activity is sensitive to the correct assembly of outer membrane proteins (porins) [121, 124, 125]. In case of events or mutations that lead to alterations in outer membrane protein (OMP) biogenesis, it is highly activated [123, 126].

On the other hand, Cpx (CpxAR) response is activated by different signals that are distinct from those that turn on σ^E-directed envelope stress [125]. These signals are closely related with genes regulating phospholipid metabolism and overexpression of membrane proteins that misfold and aggregate in

the inner membrane [125, 127]. However, Cpx and σ^E are two interconnected systems, which are both stimulated by extracytoplasmic stimuli [126]. The σ^E response is turned on by the overproduction of various outer-membrane proteins and activity of the Cpx signal transduction pathway, which consists of an inner-membrane sensor (CpxA) and a cognate response regulator (CpxR). The Cpx system is also stimulated by overproduction of the outer-membrane lipoprotein, NlpE [126].

To better understand how cells respond to changes in lipid composition, GFP-based promoter activity assays were used to monitor the gene transcription rates of some enzymes related with Cpx and σ^E-dependent stress responses (Paper I, II and IV). GFP fused reporter plasmids of promoters encoding genes for PlsB, PSD and CFA enzymes were selected from an *E. coli* promoter collection and tested if their expression levels were stimulated by acetate supplementation to LB medium (Paper I) or time-dependent overexpression of MGS (Paper II). RpoE response was also tested since RpoE levels are indicative for envelope stress [121] and peptidoglycan (envelope) synthesis is tightly coupled to phospholipid synthesis [128]. In paper IV, the influence of different lipid compositions on the Cpx response was tested using a *pcpxP-gfp* fusion transformed into various lipid clones and assayed in a similar way with paper I and II. A representative schematic illustration of the assay is given in **Figure 12**.

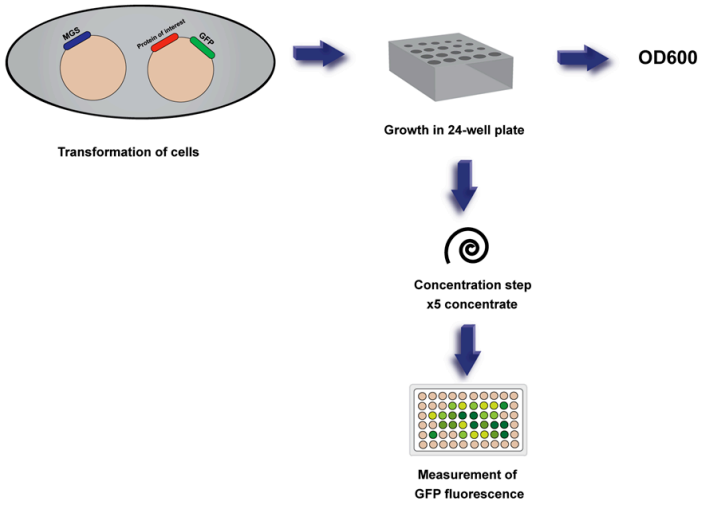

Figure 12. The experimental set-up for GFP-based promoter activity assay. BL21 AI and BL21 AI-MGS cells were transformed with two compatible plasmids (pET15b and pET28a, MGS and reporter-GFP fusion). Three colonies were selected for each transformant and grown in 24-well plates. After OD_{600} measurements, cells were concentrated five times and GFP fluorescence was measured using a fluorimeter (Excitation: 488 nm, Emission: 512 nm and Cut-off: 495 nm). Each value was normalized against previously measured OD_{600} values.

5. Summary of Papers

Paper I- Anionic lipid binding to the foreign protein MGS provides a tight coupling between phospholipid synthesis and protein overexpression in *Escherichia coli*

Candan Ariöz, Weihua Ye, Amin Bakali, Changrong Ge, Jobst Liebau, Hansjörg Götzke, Andreas Barth, Lena Mäler and Åke Wieslander

Biochemistry (2013) **52**: 5533-5544

In this study, the selective binding of anionic lipids to MGS was shown for the first time. In order to maintain the surface-associated lipids after the removal of MGS from membranes, a very gentle purification protocol was applied. Purified fractions revealed the binding of three different lipid species: anionic PG and CL lipids but also the enzymatic product of MGS, GlcDAG. Two inactive variants of MGS, E300A and E308A were observed to bind only anionic lipid species but not GlcDAG. PE as the major lipid of the *E. coli* membrane was not observed to be associated with either wild type MGS or the other inactive mutant proteins. GlcDAG binding sites were not replaced by PE, indicating a headgroup preference for GlcDAG versus PE. The specific preference for anionic lipids was also validated by the ^{31}P-NMR method using bicelles. *In vitro* observations of MGS binding to two different type of bicelle systems (-GlcDAG and +GlcDAG) indicated that the presence of GlcDAG lipid affects PG and CL binding. This finding once more endorsed a preference of MGS for GlcDAG binding.

In the second part of the study, a direct correlation between phospholipid amounts and MGS overexpression was obtained using MVDA analysis. The PLS analysis revealed a 2 fold increased phospholipid production when the lipid synthesis pathway was fed with four significant precursors/cofactors. A very good correlation was observed for all phospholipids with high concentrations of acetate and glycerol. But especially anionic lipid species (especially PG but also CL) were tightly correlated with MGS amounts produced. Due to the release of the extra material into the cytoplasm via vesicle formation, stress responses were not upregulated during this interactive cycle of lipids and membrane proteins.

Paper II- Heterologous overexpression of a monotopic glucosyltransferase (MGS) induces fatty acid remodeling in *Escherichia coli* membranes

Candan Ariöz, Hansjörg Götzke, Ljubica Lindholm, Jonny Eriksson, Katarina Edwards, Daniel O. Daley, Andreas Barth and Åke Wieslander

BBA-Biomembranes (2014) *In press*

Molecular mechanisms leading to intracellular vesicle formation in bacteria still remain enigmatic. Overexpression of the MGS protein in *E. coli* was reported to create massive amounts of vesicles and result in excessive production of phospholipids (~8 million). In Paper I, selective withdrawal of anionic lipids by MGS was thought to be the main cause triggering vesiculation but effects of MGS induction on *E. coli* membranes were not investigated.

In Paper II, the vesiculation process was monitored in a time-dependent manner using various biochemical and biophysical techniques. At first, the vesiculation was monitored by cryo-TEM and results indicated the rise of small vesicles from inner membranes at very early stages of MGS induction (between 30-60 minutes after induction). This was a striking result since the vesiculation was believed previously to take place as the result of the creation of more membrane space to accommodate the excessive molecules [77]. Of course the preproposed idea could have a role in *vesiculation*, but there must be other reasons why these vesicles are formed even at very early stages.

Membrane curvature can be created by *lipid asymmetry* [129, 130]. This could occur due to selective binding of MGS to anionic lipids, which collects them on one side of the bilayer (cytoplasmic side), thus leaving PE predominantly on the other side (periplasmic side). Moreover, fatty acid modifications can create a similar effect. In order to observe whether a possible modification takes place in membrane lipids during MGS overexpression, lipids were screened in a time-dependent manner with the TLC method. Different migration profiles indicated a modification in the lipids particularly, the PE lipid. Therefore, GC analysis was performed with purified PE lipids (and also with total lipid extracts) and we observed opposite profiles in fatty acid modifications for BL21 AI-MGS (MGS overexpressing) cells and BL21 AI (control) cells. Cyclopropanation of acyl chains (fatty acids) was lowered in BL21 AI-MGS cells whereas BL21 AI cells continued to increase their cyclopropanation during the stationary phase. In addition to cyclopropanation,

unsaturation levels were also different. Since less cyclopropanation was observed for BL21 AI-MGS cells, double bonds remain without changing into a cyclic form and this resulted in higher percentages of unsaturation. However, BL21 AI cells transformed the double bonds in their acyl chains into cyclopropanated species and thus showed lower levels of unsaturation. These findings were validated by the GFP-based promoter activity results, which showed increased transcription of the *cfa* gene in BL21 AI cells. FT-IR results revealed that MGS-expressing cells maintained a similar acyl chain ordering thus a similar membrane state by following a completely different route.

Taken together these results indicate that MGS modifies *E. coli* membranes (especially PE), which results in an asymmetric distribution of lipids in the membrane. When the selective binding of anionic lipids by MGS is also considered from Paper I, this can be proposed as the novel factor contributing to the vesiculation.

Paper III- Subcellular localization of monoglucosyldiacylglycerol synthase in *Synechocystis* sp. PCC6803 and its unique regulation by lipid environment

Tiago Toscano Selão, Lifang Zhang, Candan Ariöz, Åke Wieslander and Birgitta Norling.

PloS ONE (2014) **9** (2): e88153

Thylakoid membrane formation is dependent on lipid transport as it lacks the lipid-synthesizing machinery. There are two different theories to explain lipid transport, one proposes that the plasma membrane (PM) and the thylakoid membrane (TM) are separate entities and the lipids produced in the PM are transported to TM most likely through lipid vesicles. The other theory suggest that PM and TM are interconnected membrane systems and lipids produced in PM are transported to TM by lateral diffusion. Determining the site for lipid-synthesis is an important task since this would tell more about the synthesis and transport mechanisms. To gain insight into this subject, we monitored the localization of the monoglucosyldiacylglycerol synthase (MgdA) and its specific activity in different membrane fractions (PM1, PM2 and TM).

Western blots using a specific antibody against MgdA indicated the presence of MGS activity in three different membrane fractions but the highest MgdA localization was observed in PM1 and PM2 compared to the TM fraction. Although the TM fraction had the lowest MgdA amounts, activity results indicated that its activity was highest in this fraction. The discrepancy between higher localization of MgdA in PM but higher activity in TM could be evidence for an unknown modulator (a protein or a small molecule) present in the TM. The lipid compositions of the PM1, PM2 and TM fractions were also studied and TM was observed to have the highest sugar lipid content (GalDAG and GalGalDAG; 83 mol%) and lowest SQDAG (12 mol%) levels (data not shown).

Effects of different lipids on the activity of MgdA were studied using membrane sub-fractions (PM1, PM2 and TM) purified from *Synechocystis sp.* PCC6803 cells. The activity assays were performed with lipid-detergent mixed micelles with various compositions of lipids to be tested. Among all the lipids tested SQDAG lipid had the most dramatic effect, increasing the MGS activity seven-fold. Although the activity of the monotopic MGS (Paper I and II) is greatly increased by PG and PE lipids, the MgdA activity was slightly affected by these lipids. This result indicated that MgdA is not influenced by the curvature or charge properties of the membranes. SQDAG has a large polar head with a negative charge located at a distance from the hydrophopic part of the lipid. This unique structure is important for the activation of MgdA, thus mimicking SQDAG structure using sulphate ions with PG lipid resulted in an exacerbated activity decrease. Although SQDAG levels are low in TM, the presence of another MGS in TM stimulated by SQDAG or an unknown regulator molecule interacting strongly with sugar lipids could explain the different activities of MgdA in the PM and TM.

Paper IV- The *Escherichia coli* envelope stress sensor CpxA can sense changes in lipid bilayer properties

Rebecca Keller, Filippa Stenberg-Bruzell, Malin Burstedt, Candan Ariöz, David Wikstrom, Amelie Kelly, Åke Wieslander, Daniel O. Daley and Sabine Hunke

Manuscript

The Cpx stress system in *Escherichia coli* is an envelope stress system, which orchestrates other envelope stress responses. It is composed of three different components: an inner membrane sensor kinase (CpxA), a cyto-

plasmic response regulator (CpxR) and a periplasmic auxillary protein (CpxP). CpxA has two long transmembrane (TM) helices, a large periplasmic domain and a highly conserved catalytic domain in the cytoplasm. Under non-stress conditions, CpxA is maintained as inactive (dephosphorylated) through the binding of the CpxP dimer to its periplasmic domain. In the presence of an envelope stress, CpxP is released, CpxA is autophosphorylated and in turn, it phosphorylates the CpxR protein.

Previously, it has been shown that Cpx activity is upregulated in the AD93 strain that lacks PE lipid [131] but it is not clear whether CpxA directly senses the changes in the lipid environment or senses misfolded membrane proteins due to the changes in physicochemical properties of membrane lipids. To further investigate the influence of different lipids on the CpxAR system, the autophosphorylation and phosphotransfer activities of CpxA were tested *in vitro* and *in vivo*, using five different lipid mutants (AD93; GlcDAG, GlcGlcDAG, GalDAG, GalGalDAG).

In vitro activity assays indicated that CpxA autophosphorylation activity is enhanced by increased membrane curvature but decreased with higher anionic lipid (PG and CL) content. In contrast, CpxA phosphotransfer activity was negatively affected by increased membrane curvature and PG had a positive regulation on CpxA. However, CL was observed to have inhibitory effects. Additionally, membrane thickness was observed to be an important factor for CpxA activity and was optimal for natural lipids with 16.5 carbons. These results showed that CpxA senses bilayer properties directly through lipid binding and thus excludes the possibility of sensing misfolded proteins. *In vivo* assays also validated *in vitro* results but also indicated the role of Mg^{2+} ions to change lipid shapes and thus the curvature type created in the bilayer.

6. Concluding remarks and future perspectives

In this PhD thesis, the interplay of lipids and membrane proteins were studied using 3 different types of membrane proteins and a multidisciplinary approach. We have chosen all our model membrane proteins with respect to their different chemical properties, functional roles and ways of interaction with the membrane (peripheral and integral). All model membrane proteins reacted differently to different lipid environments due to their distinct structural features and needs. Although MGS and MgdA are two close homologue proteins from two distinct species, their responses to different types of lipids were different. During my PhD, I observed that the regulation of each lipid in the membrane might differ for different kinds of proteins. Anionic lipids (PG and CL) and a nonbilayer-prone lipid (PE) have positive effects on MGS activity, whereas they had minor effects on MgdA activity (Paper III). In addition to the effects of individual lipid molecules, physicochemical properties of the whole bilayer (surface charge, fluidity, membrane curvature, bilayer thickness *etc*.), is also an important determinant for the proper functioning of membrane proteins as was observed for CpxA sensor in Paper IV.

In Paper II, we have also observed that a membrane protein (MGS) interferes with the lipid synthesis and can regulate all these aforementioned properties. It is known that high membrane fluidity promotes protein activity by reducing viscosity, which stimulates the activities and increases the diffusion ability of membrane proteins. Additionally, creation of higher membrane elasticity by adjusting membrane fluidity allows the bilayer to embed membrane proteins optimally during their overexpression [132].

There is a general belief that lipids act only as cofactors helping proteins to fold and function properly. Besides this, they can also force various membrane proteins to assemble into specialized domains and thus create the correct micro-environment to achieve the highest efficiency [133]. Additionally, membrane proteins can also regulate the properties of membrane lipids through changing the lipid-bilayer properties. Two different examples besides MGS in Paper II, are matrix proteins (M protein) from Newcastle disease virus [134] and the actin homologue MreB [132]. All of these proteins are peripheral and associated to the bilayer by an N-terminal amphipathic helix [132, 135]. They are capable of regulating membrane fluidity by changing fatty acid compositions and creating more fluid proteolipid domains for themselves to be optimally inserted into the membrane. These fluid domains are usually suggested to have roles in budding and vesicle formation [134]. The mechanism leading to the vesiculation can also be re-

lated with proteolipid interactions within a fluidlike microenvironment and is not only based on the intrinsic topology, molecular shape of the protein lattice or intrinsic curvature created by non-bilayer lipids (PE). All these factors stated above may contribute to drive membrane curvature formation.

Further work is required to gain insight into the vesiculation ability of these proteins and reveal the mechanisms that induce local curvatures. This could be done by the analysis of various peripheral membrane proteins selected with the stated characteristics and monitoring their effects on the fatty acid and lipid compositions with respect to their abilities to form intracellular membrane structures. Moreover, their regulation on the activities of other membrane proteins should also be taken into consideration. This is an important task for understanding many fundamental cellular processes such as formation of organelles, cell division, endocytosis, synaptic vesicle fusion, molecular trafficking and signaling [136-138]. It is generally accepted that the intrinsic shapes of some curvature mediating proteins and their oligomers that result in membrane crowding on the bilayer surface forces the membrane to curl. Here, we have revealed that not only these factors mediate curvature formation, but the interplay of membrane proteins with lipids is responsible for the remodeling of cellular membranes.

7. Populärvetenskaplig sammanfattning på svenska

Samspelet mellan lipider och membranproteiner är känt att påverka membranprotein topologi och har stor betydelse för kontroll av deras funktioner. I denna avhandling, studerades lipidernas påverkan på funktionen av membranproteiner genom att använda tre olika membranproteinmodeller.

Ett monotopisk membran protein, monoglucosyldiacylglyecerol syntas (MGS) från *Acholeplasma laidlawii* är känd för att inducera dem intracellulära vesiklarna när den är utryckt i *Escherichia coli*. Mekanismen bakom detta ovanliga fenomen undersöktes med olika biokemiska och biofysiska tekniker. Resultaten visade en fördubbling av lipidsyntes i cellen, som utlöstes av selektiv bindning av MGS på anjoniska lipider (PG och CL). Multivariat data analys (MVDA) visade en god korrelation med produktionen av MGS. Vidare, förmånliga anjonisk lipid sekvestrering av MGS visat sig inducera en annan fettsyra modellering av *Escherichia coli* membran. Rollerna som en specifik lipidbindning spelar och den troliga mekanismen som leder till intracellulär vesikel bildning utreddes också.

En homolog av MGS från *Synechocystis* sp. PCC6803 valdes som den andra modellen. MgdA är ett integralt membran protein med flera transmembran helixar och en unik membrantopologi. Olika typer av lipiders inverkan på MgdA aktivitet testades med olika membranfraktioner från *Synechocystis*. Resultaten visade en mycket tydlig profil jämfört med *Acholeplasma laidlawii* MGS. En anjonisk lipid SQDG konstaterades vara den arten av membranet som ökar MgdA aktivitet 7-faldigt, medan PG endast hade små effekter på MgdA. Dessutom föreslogs en fungerande modell av MgdA för biosyntesen och flödet av sockerlipider mellan *Synechocystis* membran.

Det sista modellsystemet var ett annat integralt membranprotein med en distinkt struktur, men också med en annan funktion. Kuvertet spänningssensor CpxA och dess samverkan med *E. coli* membran studerades. CpxA autofosforyleringsaktivitet visade sig vara positivt reglerad av fosfatidyletanolamin (PE) och negativt reglerad av anjoniskt lipid innehåll. Däremot fosforylering av CpxR genom CpxA avslöjade ökas med PG, men hämmas av CL. Icke-dubbelskikts lipider hade en negativ inverkan på CpxA fosfotransfer aktivitet.

Sammantaget ger dessa studier en bättre förståelse för betydelsen av samspelet mellan lipider och modellmembranproteiner, som diskuterats här.

8. Türkçe Özet

Lipidlerin membran proteinleri ile olan karşılıklı etkileşimleri membran proteinlerinin yapılarını, fonksiyonlarını ve aktivitelerini düzenlemektedir. Bu doktora tezinde, lipidlerin membran proteinlerinin yapı ve fonksiyonlarındaki düzenleyici etkileri üç farklı model membran proteini kullanılarak çalışılmıştır.

Acholeplasma laidlawii kaynaklı monotopik (membran yüzeyinde oturan) bir membran proteini olan monoglukosildiaçilgliserol sentaz (MGS)'ın *Escherichia coli*'de üretildiğinde hücre içi vesiküler oluşumlara yol açtığı bilinmektedir. Bu sıradışı olaya yol açan mekanizma çeşitli biyokimyasal ve biyofiziksel yöntemler kullanılarak araştırılmıştır. Sonuçlar MGS proteinin anyonik lipidlere (PG ve CL) seçici bir şekilde bağlanması ile hücre içindeki lipid miktarının iki katına çıktığını ortaya koymuştur. Çok değişkenli data analizi de bu olayın yüksek orandaki MGS üretimi ile doğru orantılı olduğunu göstermektedir. Bununla birlikte, MGS'ın seçimli olarak anyonik lipidlerle etkileşiminin *E. coli* membranında daha farklı bir yağ asidi kompozisyonuna yol açtığı görülmüştür. Bu spesifik lipid-bağlanmasının hücre içi vesiküler oluşum mekanizmasına olan etkisi de incelenmiştir.

İkincil model olarak, MGS proteinin *Synechocystis* sp. PCC6803' teki bir homoloğu seçilmiştir. MgdA, membranı birden fazla şekilde geçen heliksli yapıya sahip integral bir membran proteinidir. *Synechocystis* lipidlerinin MgdA proteini üzerine olan etkisi, bu proteini içeren farklı membran fraksiyonları kullanılarak test edilmiştir. Sonuçlar, *Acholeplasma laidlawii* MGS'inden çok daha farklı bir profil göstermiştir. Bir anyonik lipid olan PG'ün MgdA aktivitesine önemli bir etkisi olmadığı gözlemlenirken bir başka anyonik lipid olan SQDG'ün MgdA aktivitesini 7-kat arttırdığı gözlemlenmiştir. Buna ek olarak, MgdA' ın gluko ve galaktolipidlerin biyosentezindeki görevi ve bu lipidlerin *Synechocystis* membranları arasındaki akış mekanizması için bir model öne sürülmüştür.

Son model system olarak ise farklı bir yapıya ve fonksiyona sahip bir başka integral membran proteini seçilmiştir. *E. coli* hücre çeperi stres sensörü olan CpxA'nin hücre zarı ile olan etkileşimi çalışılmıştır. CpxA otofosforilasyon aktivitesinin fosfatidiletanolamin (PE) tarafından pozitif ama anyonik lipidler tarafından negatif olarak etkilendiği bulunmuştur. Buna karşın, CpxA'nın fosfotransfer aktivitesinin PG tarafından arttırıldığı ve CL tarafından inhibe edildiği ortaya konmuştur. Bilayer yapıda olmayan lipidlerin ise CpxA fosfotransfer aktivitesine negatif bir etkisi olduğu görülmüştür.

Sonuçta, bu çalışmalar membran lipidleri ve bu tezde model olarak seçilen membran proteinleri arasindaki etkileşimler hakkında daha geniş bir bilgiye sahip olmamızı sağlamıştır.

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