Membrane interactions of glycosyltransferases

Licenciate thesis presented by

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Abstract

Many important biological processes occur near or in membranes. The role of membranes is not merely confined to compartmentalization, they also form the matrix for membrane associated proteins and are of functional importance. Membrane associated proteins on the other hand require specific membrane properties for proper function. The interactions between membranes and proteins are thus of paramount importance and are at the focus of this work.

To draw valid conclusions about the nature of such interactions the membrane mimetics required in biophysical methods must faithfully mimic crucial properties of biological membranes. To this end, new types of small isotropic bicelles which mimic plant and bacterial membranes were characterized by their size and lipid dynamics using solution-state NMR. Small isotropic bicelles are specifically well suited for solution-state NMR studies since they maintain a bilayer while being sufficiently small to conduct interpretable experiments at the same time. Monogalactosyl diacylglycerol and digalactosyl diacylglycerol, which are highly abundant in thylakoid membranes, were successfully incorporated into bicelles. Also, it was possible to make bicelles containing a lipid mixture extracted from *Escherichia coli* cells.

A fundamental physical property of lipids in bilayers is their phase behaviour and thus the dynamics that lipids undergo in a membrane. Here, the dynamics of $^{13}$C–$^{1}$H bonds in lipids were studied by nuclear spin relaxation. From such studies it was found that the glycerol backbone of lipids in bicelles is rigid while the flexibility of the acyl chain increases towards its end. Bulky head groups are rigid, while smaller head groups are more dynamic than the glycerol backbone. Acyl chain modifications, like unsaturations or cyclopropane moities, that are typically found in *E. coli* lipids, locally increase the rigidity of the acyl chain.

Membrane interactions of a putative membrane anchor of the glycosyltransferase WaaG, MIR-WaaG, were studied by fluorescence methods, circular dichroism and solution-state NMR. It was found that MIR-WaaG binds to vesicles that mimic the anionic charge of *E. coli* inner membranes and that α-helical structure is induced upon interaction. The NMR-structure of MIR-WaaG agrees well with the crystal structure and from paramagnetic relaxation enhancement studies it could be concluded that a central part of MIR-WaaG is immersed in the membrane mimetic. Based on these results a model of the membrane interaction of WaaG is proposed where MIR-WaaG anchors WaaG to the cytosolic leaflet of the *E. coli* inner membrane via electrostatic interactions. These are potentially enhanced by membrane interactions of Tyr residues at the membrane interface and of hydrophobic residues inside the membrane.
Abbreviations

CD   Circular dichroism
CL   Cardiolipin
CMC  Critical micelle concentration
CSA  Chemical shift anisotropy
DD   Dipole-dipole
DGDG Digalactosyl diacylglycerol
DHPC 1,2-dihexanoyl-sn-glycero-3-phosphatidylcholine
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine
DPC  Dodecylphosphocholine
Gd(DTPA-BMA) Gd$^{3+}$ diethylenetriamine pentaacetic acid - bismethylamide
LPS  Lipopolysaccharide
LUV  Large unilamellar vesicle
MGDG Monogalactosyl diacylglycerol
NOE  Nuclear Overhauser enhancement
PE   Phosphatidylethanolamine
PG   Phosphatidylglycerol
POPC 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
POPG 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
PRE  Paramagnetic relaxation enhancement
SQDG Sulfoquinovosyl diacylglycerol
1 List of Publications


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2 Introduction

In this thesis novel membrane models applicable to solution-state NMR that mimic *Escherichia coli* and plant thylakoid membranes and the membrane interaction of the *E. coli* glycosyltransferase (GT) WaaG are described. The studies were performed using a wide array of biophysical techniques, which are described in chapter 3. A discussion of the results and a conclusion are presented in chapter 4 and 5.

2.1 Lipid membranes in vivo and in vitro

Biological membranes are highly complex lipid bilayers that fulfill a multitude of functions. In order to draw valid conclusions from biophysical experiments, biological membranes need to be simplified. At the same time, the simplified models have to maintain the properties of interest. Some key features of bacterial inner membranes and plant thylakoid membranes are discussed and membrane systems that at the same time mimic the characteristics of such membranes and are amenable to biophysical methods are presented.

2.1.1 Biological membranes of plants and bacteria

Biological membranes are required for compartmentalization of cells and organelles. Apart from their structural importance, membranes have functional roles as well [1, 2]. For example, phosphatidylethanolamine (PE) in *E. coli* inner membranes has chaperone-like functions in that it is required for correct membrane insertion of LacY [3]. Moreover, PE-deficient cells show substantial membrane related deficiencies, e.g. in cell division [4]. The root of these malfunctions may be an enrichment in negative charge density and an alteration of curvature stress in PE-deficient strains [4]. Similarly, phosphatidylglycerol (PG) and cardiolipin (CL) are required for a myriad of membrane-related processes, e.g. maintenance of a permeability barrier, protein translocation and cell growth [2]. Finally, some proteins use lipids as structural or functional co-factors. For instance, galactolipids are found in the crystal structure of photosystem II and may be required for correct assembly and/or function [5, 6].

*E. coli* inner membranes mainly consist of three types of lipid head groups: PE, PG, and CL. PE is most abundant (75%), followed by PG (15%), and CL (5%) [1, 7–9]. The acyl chain composition is more diverse and depends on external conditions. Under unstressed conditions 16:0 acyl chains are most abundant (40%) and the ratio of unsaturated to saturated acyl chains is about 0.4 – 0.5 [1, 7, 10]. Notably, a substantial part of unsaturated acyl chains contains a cyclopropane moiety for bacteria in the stationary phase. A reason might be that cyclopropane moieties increase the physico-chemical stability of membranes [11, 12]. The variability in head group and acyl chain types is required to maintain a fluid membrane under varying conditions, for lateral sorting of lipids and proteins and for a large array of cellular processes, e.g. cell division, which require morphological changes of the bilayer [1].

The glycolipids monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfogalactosyl diacylglycerol (SQDG) constitute about 60% of thylakoid membranes in plants under unstressed conditions [5]. MGDG is a non-bilayer forming lipid and, similar to PE, it has been speculated that the curvature stress it imposes on membranes is required for proper function of membrane-associated processes and proteins [11, 13, 14]. Upon phosphate starvation the synthesis of glycolipids is upregulated in many bacteria and plants. For example,
in *Arabidopsis thaliana* the content of DGDG and SQDG increases under phosphate stress and thylakoid membranes consist up to 80% of glycolipids [5, 6].

### 2.1.2 Membrane mimetics for biophysical applications

*In vitro* studies of protein-membrane interactions require that membrane mimetic systems be artificially produced. A broad range of membrane mimetic systems is available of which micelles, isotropic bicelles, and large unilamellar vesicles (LUVs) are relevant within the scope of this work.

LUVs are spherical lipid bilayers that are about 100 nm in diameter. Therefore, they adequately mimic the low curvature of cell membranes. Moreover, the lipid composition is variable and can be adapted to the question at hand [15, 16]. In order to achieve high-resolution spectra, small membrane mimetics are required in solution-state NMR. Therefore, detergent micelles are commonly used membrane mimetics and structural as well as dynamic studies are frequently conducted in such systems [8, 17]. However, it has been shown that the high surface curvature of micelles can distort protein structure and function [18–25]. More realistic membrane properties are conferred by small isotropic bicelles that according to the ideal bicelle model contain a central disk-shaped lipid bilayer stabilized by detergents in the rim [26–28]. The morphology and size of bicelles depends on the q-value, the molar ratio of lipids to detergent [26, 29]. In the range from $q = 0.25 - 1$ bicelles resemble the ideal bicelle model, although the degree of detergent-lipid segregation is still a matter of debate [27, 30]. For $q < 0.25$ detergent and lipids mix forming mixed micelles [24]. For $q > 1$ sheet-like structures perforated by pores lined with detergents were observed. Under appropriate conditions those structures align in an external magnetic field while isotropic bicelles do not [31, 32].

Typical isotropic bicelles are about 10 nm in diameter and 4 nm thick [27]. Apart from the q-value, temperature [27, 30, 33], pH [34], and total amphiphile concentration [35] affect their size. The lipid composition in bicelles is flexible. Sphingomyelin [36], anionic lipids [37, 38], galactolipids [39], lipids extracted from *E. coli* [40] and lipids of varying acyl-chain length [41] and degree of unsaturation [39, 42, 43] have been incorporated into bicelles. Since bicelle formation requires the detergent concentration to be significantly above its critical micelle concentration (CMC), detergents of low CMC have been employed to make bicelles [35].

Membranes and lipids in membranes undergo a wide range of dynamics (Fig. 2.1) on a broad range of timescales [44, 45]. In this study $^{13}$C–H picosecond bond dynamics as well as lipid reorientation and membrane protrusion which occur on a picosecond to nanosecond timescale are investigated by the methods described in section 3.3.1.

### 2.2 Membrane-associated proteins

Proteins associate with membranes in a variety of ways (Fig. 2.2): Integral membrane proteins span the entire bilayer with one (bitopic) or several (polytopic) transmembrane α-helices. Also, β-barrel proteins are integral membrane proteins. Monotopic membrane proteins are permanently anchored to one leaflet of the bilayer. This anchoring can be achieved by acyl chains covalently bound to the protein or by electrostatic and hydrophobic interactions between the membrane and the protein, e.g. through amphiphilic helices. Peripheral membrane proteins bind reversibly to membranes, e.g. by structural changes which expose a hydrophobic domain or by electrostatic and hydrophobic interactions with the membrane or membrane proteins [7].
Figure 2.1: Lipid and membrane dynamics. Lipids rotate and protrude the membrane on a ps-ns timescale and $^{13}\text{C}^{-1}\text{H}$ bond dynamics occur on a ps timescale. Dynamics in membranes are typically slower. The picture is taken from [46] and was adapted from [44, 45].

Figure 2.2: Membrane associated proteins. Red: Cytochrome c (PDB: 351C) is a peripheral membrane protein. Green: The carbonic anhydrase IV (PDB: 1ZNC) is a monotopic protein binding to one leaflet of the membrane via a glycosylphosphatidylinositol-anchor indicated in black [47]. Orange with yellow $\beta$-sheets: The glycosyltransferase WaaG (PDB: 2IW1) is believed to be a monotopic membrane protein, binding to one leaflet of the membrane via electrostatic and hydrophobic interactions. Blue: The outer membrane protein OmpX (PDB: 1QJ8) is an integral-membrane $\beta$-barrel protein. Purple: Proteorhodopsin (PDB: 2L6X) is an integral-membrane $\alpha$-helical protein. The bilayer was generated with CHARMM [48–52].
2.3 Monotopic GT-B glycosyltransferases

Glycosyltransferases (GTs) form a vast class of proteins that catalyze the formation of glycosidic bonds. Although the Carbohydrate Active Enzymes database (CAZy, www.cazy.org [53, 54]) lists about 200 000 GTs, only 3 distinct folds have been identified whereof most GTs of known structure adopt either a GT-A or a GT-B fold [55, 56]. A third fold, GT-C, has recently been identified [57, 58] and some GTs do not adopt any of the three folds [59].

The structures of 22 membrane-associated GT-B GTs have been solved, about half of which are believed to be monotopic membrane proteins [60]. The GT-B fold is characterized by a Rossmann-fold, consisting of alternating $\beta$-strand and $\alpha$-helix segments where the $\beta$-strands form a $\beta$-sheet wrapped by $\alpha$-helices. This motif is typically found in nucleotide binding proteins [61]. GT-B type GTs consist of two such domains (see WaaG in Fig. 2.2 and Fig. 4.4). The active site is located in the cleft between the N- and C-terminal domain [60].

Based on the study of the monotopic GTs AlMGS and AtDGD2 a model of the membrane interaction of monotopic GT-B GTs was suggested by Ge and coworkers [62] and recently reviewed by Albesa-Jové and coworkers ([60] and references therein). In this model monotopic GT-B GTs permanently anchor to one leaflet of the lipid bilayer with their N-terminal domain. This tight association is maintained through electrostatic interactions between anionic lipids and cationic side chains. Moreover, Trp and Tyr residues located at the bilayer interface seem to be involved in binding as well as hydrophobic amino acids that are immersed into one leaflet. The C-terminal domain is assumed to be more flexible so as to accommodate donor and acceptor molecules in the active site. Experimental evidence for this is, however, lacking. Moreover, with the exception of MGS ($K_D$ on the order of pM [14, 63]) membrane association constants are unknown such that it is unclear whether or not the association is permanent in vivo.
3 Methods

3.1 Circular dichroism

Circular dichroism arises from the difference in absorption of left- and right-handed circularly polarized light by a chromophore, which is either chiral itself, achiral but covalently bound to a chiral center, or located in an asymmetric environment [64–66]. Quantum mechanically circular dichroism is described by a non-zero rotational strength $R$

$$\Delta \varepsilon \propto R = \Im \left\{ \langle \Phi_f | m | \Phi_i \rangle \langle \Phi_i | \mu | \Phi_f \rangle \right\} \neq 0$$ (3.1)

which means that the CD-signal $\Delta \varepsilon$ is only non-zero if the magnetic and electric dipole moment operators $m$ and $\mu$ are non-zero and non-perpendicular. These conditions are fulfilled for chromophores which do not have a center or a plane of symmetry or for which these symmetries are broken due to a chiral environment [64–66].

In proteins the dominant chromophore in the far-UV spectrum (180 – 250 nm) is the peptide bond with a $\pi \rightarrow \pi^*$ transition at 190 nm and a $n \rightarrow \pi^*$ transition at 220 nm. The peptide bond has to a good approximation planar symmetry and is therefore inherently achiral [64] but secondary structure elements give rise to distinct asymmetric environments and thus display characteristic features in far-UV CD-spectra [67]. Since the geometry of the polypeptide backbone uniquely defines the secondary structure of the protein, far-UV CD of the peptide bond provides a quick method to determine the secondary structure content of proteins.

3.2 Fluorescence spectroscopy

Upon absorption of a photon, fluorescent molecules are excited from the $S_0$ singlet electronic ground state to some vibrational state of a higher electronic state. Within picoseconds, molecules relax to the lowest vibrational level of the first excited electronic state $S_1$ and then return to a vibrational state of $S_0$ within nanoseconds either emitting a photon or via non-radiative pathways. By altering the weight of radiative versus non-radiative relaxation pathways fluorescence measurements allow conclusions on the localization of fluorophores. Moreover, typical fluorescence lifetimes are on the order of nanoseconds, which means that dynamics on that timescale are observable. Aromatic amino acid residues in proteins are fluorescent and can thus be used as intrinsic probes [68].

In this study, hydrophobic and hydrophilic quenchers were used to probe the membrane association of Tyr residues in a membrane anchor of the glycosyltransferase WaaG. The parallax method was employed to quantify the immersion depth of these residues in the membrane. The dependence of the membrane binding of the anchor on surface charge density was probed by fluorescence anisotropy and the interaction was quantified with fluorescence measurements of the dissociation constant $K_D$.

3.2.1 Quenching

Fluorescence quenching is the reduction in emission intensity of a fluorophore due to its proximity to a quencher by non-radiative processes. It can thus be used to determine the accessibility of a quencher to a fluorophore. Hence, by introducing quenchers in different parts of the bilayer or the aqueous environment the localization of a protein or a peptide can be determined. Typical
quenchers are acrylamide and paramagnetic species.

Dynamic quenching occurs when a fluorophore returns to its ground state upon collision with the quencher during the lifetime of the excited state. This effect is described by the Stern-Volmer equation [69]:

$$\frac{I_0}{I} = 1 + K_{SV}[Q]$$

(3.2)

where $I_0$ and $I$ are the fluorescence intensities in the absence of quencher and at quencher concentration $[Q]$, respectively, and $K_{SV}$ is the Stern-Volmer quenching constant.

Static quenching occurs when a non-fluorescent complex is formed between fluorophore and quencher. The mathematical description of static quenching is identical to Eq. 3.2 i.e. it is not possible to determine whether quenching is dynamic or static from fluorescence intensity measurements. To resolve this question, fluorescence lifetimes need to be measured in the absence and presence of quencher. If both dynamic and static quenching occur, higher order terms in $[Q]$ need to be introduced into Eq. 3.2 [70].

### 3.2.2 The parallax method

The parallax method allows determination of the immersion depth of a fluorophore into lipid membranes. The decrease in fluorescence intensity in the presence of quenchers located at different, known positions within the bilayer is measured and the fluorophore distance from the bilayer center $z$ is evaluated as:

$$z = L_{c1} + \frac{L_{21}^2 - \frac{\ln(I_1/I_2)}{2L_{21}}}{L_{21}}$$

(3.3)

where $L_{c1}$ is the distance of the shallow quencher from the bilayer center, $L_{21}$ is the distance between shallow and deep quencher, $C$ is the quencher concentration in the membrane plane, and $I_1$ and $I_2$ are the fluorescence intensities in the presence of the shallow and deep quencher, respectively.

Depending on the expected depth of the fluorophore, typical quenchers used are (2,2,6,6-tetramethylpiperidin-1-yl)oxyl-PC (TEMPO-PC) with a radical in the head group and 5- and 10-doxyl PC with radicals at position 5 or 10 in the acyl chain. While lipid dynamics during the excitation lifetime have little effect on the result, the distance between fluorophore and quencher must not exceed the quenching radius (typically 7 Å) to obtain a reliable estimate of $z$ [71–73].

### 3.2.3 Anisotropy

Fluorescence anisotropy reports on the depolarization of emitted light by fluorophores that were selectively excited by polarized light. The principal cause of depolarization is due to rotational diffusion of fluorophores during the excitation lifetime. However, this process may be obscured by depolarization through resonance energy transfer or scattering. Fluorescence anisotropy is defined as [68]:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

(3.4)

where $I_\parallel$ and $I_\perp$ are the emitted fluorescence intensity with the emission polarizer oriented parallel or perpendicular to the excitation polarizer.

Polarized light preferentially excites fluorophores that have their absorption dipole oriented parallel to the direction of the electric field vector and the absorption probability is proportional to $\cos^2 \phi$, where $\phi$ is the angle between the electric field vector and the absorption dipole. In isotropic solutions where no depolarization processes occur (e.g. dilute, viscous solutions) and the absorption and emission dipoles of the fluorophore are colinear the maximum anisotropy is $r = 0.4$. In general, the fundamental anisotropy $r_0$, i.e. the anisotropy in the absence of
depolarization processes, of a fluorophore is lower than that value if absorption and emission
dipoles are displaced by an angle $\beta$ \[68\]:

$$r_0 = \frac{2}{5} \frac{3 \cos^2 \beta - 1}{2} \quad (3.5)$$

The fundamental anisotropy of tyrosine residues was determined to be $\sim 0.3$ \[74\]. This value
can be significantly decreased by resonance energy transfer if Tyr residues are in close spatial
proximity. For neighbouring Tyr residues $r = 0.19$ has been reported \[75\].

### 3.2.4 Measurements of the dissociation constant $K_D$

The affinity of a protein or peptide for membranes can be quantified by the equilibrium disso-
ciation constant $K_D$ \[76\]. When the total lipid concentration equals $K_D$ half of the protein is
bound to membranes. From $K_D$ thermodynamical properties of a system can be determined via
the Gibbs free energy \[77\].

Assuming a single binding site the increase in fluorescence intensity upon titration of lipids
into the peptide solution is described by \[78\]:

$$\frac{I - I_0}{I_0} = \frac{B_{\text{max}} [L]}{K_D + [L]} \quad (3.6)$$

where $I_0$ is the initial fluorescence intensity, $I$ is the fluorescence intensity at lipid concentration
$[L]$, and $B_{\text{max}}$ is the maximum enhancement of the normalized fluorescence intensity, i.e. when
all protein is bound \[73\].

### 3.3 NMR

The phenomenon of nuclear magnetic resonance is based on the spin property of nuclei. When
an NMR-active nucleus is placed in an external magnetic field the degeneracy of the spin en-
ergy levels is lifted and electromagnetic radiation can be absorbed. The absorption frequency is
proportional to the external magnetic field and the proportionality constant, the magnetogyric
ratio, is a property of the nucleus. Since signal intensities depend on the small population dif-
ferences of spin energy states, NMR is a low-sensitivity method. Moreover, the natural abundance
of NMR-active nuclei like $^{13}\text{C}$ and $^{15}\text{N}$ is 1 % and 0.4 %. Therefore, NMR experiments often
require isotope labeling and/or mM concentrations.

Among the nuclear properties that can be determined by NMR are resonance frequencies and
relaxation rates of spins. Those properties depend on the local magnetic environment which
in turn is influenced by structure and dynamics of the molecule under investigation. Molecular
structure and dynamics can thus be probed on an atomic scale.

Here, the NMR-structure of a membrane anchor of the glycosyltransferase WaaG was solved
and its localization in micelles was studied by paramagnetic relaxation enhancement using a
soluble lanthanide complex. Diffusion and relaxation measurements were employed to charac-
terize the size of and lipid dynamics in novel bicelle systems that incorporate galactolipids or
lipid extracts from \textit{E. coli}.

#### 3.3.1 Dynamics

In NMR dynamical processes mediate relaxation of non-equilibrium states through various
anisotropically interactions. For systems with coupled spins, such as $^{13}\text{C}^\text{-}\text{H}$ bonds in organic
molecules, like lipids or proteins, the dominant relaxation mechanism is mediated through dipole-
dipole (DD) interactions. Molecular dynamics of appropriate frequencies that modulate these
anisotropic interactions randomly return non-equilibrium magnetization to the ground state. The mechanism is analogous to the intentional generation of transverse magnetization by radio-frequency pulses of appropriate frequencies. The spectral density function $J(\omega)$ describes the distribution of frequencies that occur due to molecular motion. When DD relaxation is the dominating relaxation process, the longitudinal relaxation rate $R_1$ and the nuclear Overhauser enhancement (NOE) in terms of $J(\omega)$ are given by [79, 80]:

$$R_1 = d^2 \left[ J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C) \right] \text{ with } d = \frac{\mu_0 h \gamma H \gamma C}{8\pi^2 \nu^3} \quad (3.7)$$

$$\text{NOE} = 1 + \frac{\gamma H}{\gamma C} \left[ \frac{6J(\omega_H + \omega_C) - J(\omega_H - \omega_C)}{J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)} \right] \quad (3.8)$$

where $\omega_C$ and $\omega_H$ are the Larmor frequencies of $^1H$ and $^{13}C$, $\mu_0$ is the vacuum permeability, $h$ is Planck’s constant, $\gamma_H$ and $\gamma_C$ are the magnetogyratory ratios and $r$ is the $^{13}C$-$^1H$ bond length.

In principle, $J(\omega)$ can be expressed in terms of molecular dynamics but the functional form is complex. In a model-free approach suggested by Lipari and Szabo [81] and Wennemström [82, 83], molecular dynamics are described in terms of a squared order parameter, or amplitude of motion, $S^2$, and a rotational correlation time, $\tau$. In the specific case of lipid motions in bicelles, the $^{13}C$-$^1H$ bond dynamics as well as the dynamics of the entire lipid occur on timescales that contribute to spin relaxation (see Fig. 2.1). Importantly, both types of motions occur on different timescales and are therefore independent of each other. Thus, bicelle dynamics are described by four parameters: $S^2_{loc}$, $\tau_{loc}$, $S^2_{lip}$, and $\tau_{lip}$. The spectral density function is given by [44, 84, 85]:

$$J(\omega) = \frac{2}{5} \left[ \frac{S^2_{loc} - S^2_{loc} S^2_{lip}}{1 + \omega^2 \tau_{lip}} \tau_{lip} + \frac{1 - S^2_{loc}}{1 + \omega^2 \tau_T} \tau_T \right] \quad \text{ with } \tau_T = \frac{\tau_{lip}\tau_{loc}}{\tau_{lip} + \tau_{loc}} \quad (3.9)$$

Therefore, by measuring a sufficiently large set of relaxation rates, dynamical properties of $^{13}C$-$^1H$ bond vectors in lipids and of the entire lipid within a bicelle can be determined.

### 3.3.2 Structure determination

The NOE is effective through space and has a $r^{-6}$ distance dependence. Even though a strict quantitative interpretation of NOE intensities is not possible, molecular structures can be obtained from a sufficiently large set of qualitative, i.e. not too rigid, distance constraints [80]. For small peptides or proteins below $\sim 10$ kDa such distance constraints are obtained from 2D-NOESY experiments [87, 88]. Furthermore, $H^\alpha$, $C^\alpha$, and $C^\beta$ secondary chemical shifts are sensitive to the secondary structure of a protein, i.e. they can be used to derive dihedral angle constraints [89, 91].

Using a set of constraints a set of molecular structures are obtained from in silico methods (e.g. CYANA) which generate structures that try to fulfill all constraints simultaneously and are ranked by the degree of constraint violation evaluated by an energy function [92].

### 3.3.3 Paramagnetic relaxation enhancement

Paramagnetic relaxation enhancement (PRE) is widely used in NMR-applications to obtain structural and dynamic information. Here, the discussion is limited to PREs induced by paramagnetic species which are not covalently bound to proteins. The proximity of paramagnetic species enhances spin relaxation rates. The effect is proportional to $r^{-6}$ [93, 94]. For Mn$^{2+}$, Gd$^{3+}$, and nitroxide radicals electron spin relaxation is relatively slow and thus PRE is predominantly driven by the Solomon relaxation mechanism which in turn is insensitive to cross-relaxation effects [93, 94]. Therefore, PRE effects are reliably measured with those paramag-
netic species. In general, the use of Gd-chelates, like Gd$^{3+}$ diethylenetriamine pentaacetic acid - bismethylamid (Gd(DTPA-BMA)), is preferred over Mn$^{2+}$ since the former is inert towards electrostatic or hydrophobic interactions while Mn$^{2+}$ preferentially binds to anionic molecules or side chains. Experimentally, PREs ($\Gamma_{1,2}$) are measured as the difference in relaxation rates in the paramagnetic and diamagnetic system:

$$\Gamma_i = R_{i,\text{para}} - R_{i,\text{dia}} \text{ with } i = 1, 2$$ (3.10)

While it is advisable to measure actual relaxation rates this may not be feasible for e.g. $^{13}$C in natural abundance samples. In such cases peak intensity reductions due to the proximity of paramagnetic species may be taken as a proxy for relaxation rate measurements [94].

### 3.3.4 Translational diffusion

Translational diffusion experiments report on the hydrodynamic radius as well as on the association of molecules. Transverse magnetization is generated and the position of spins along the external magnetic field is encoded by a magnetic field gradient of length $\delta$. Following another 90° pulse, molecules diffuse during a delay time $\Delta$. At the end of the delay a refocussing pulse is applied and the spin positions are decoded by another gradient pulse. In the absence of diffusion the signals are completely refocussed. However, in the presence of diffusion, the signal intensity is decreased [97, 98]. The loss in signal intensity is described by Stejskal-Tanner’s equation [99–101]:

$$I = I_0 \exp \left( -D\gamma^2\delta^2 \left( \Delta - \frac{1}{3}\delta \right) g^2 \right)$$ (3.11)

where $I_0$ and $I$ are the signal intensities at zero gradient and at gradient strength $g$, respectively, $\gamma$ is the magnetogyratic ratio, $\delta$ and $\Delta$ are as defined above and $D$ is the translational diffusion coefficient.

Diffusion coefficients can be used to derive information about molecular size. This has been extensively used to investigate bicelle formation and to determine the extent of binding of e.g. peptides to bicelles [102].
4 Results and Discussion

4.1 Bicelles mimicking plant and bacterial membranes

In order to more accurately study the lipid interaction of membrane-associated proteins or peptides by solution-state NMR, we characterized bicelles containing lipid mixtures that more faithfully mimic plant and bacterial membranes than commonly used bicelle systems. Four types of bicelles were studied: Bicelles containing the galactolipids MGDG and DGDG (Paper I) and bicelles containing lipids extracted from two E. coli strains (Paper II). The hydrodynamic radii of bicelles were obtained from diffusion measurements and lipid dynamics were characterized by dynamic parameters obtained from the model-free approach (Eq. 3.9) using relaxation parameters measured at two different magnetic field strengths.

Up to 30 mol % of DMPC could be replaced by either of the galactolipids. Since galactolipids and DMPC diffused at the same rate, we conclude that they partition into the same assembly. The diffusion coefficients of galactolipid bicelles were lower than for the reference DMPC/DHPC bicelles, i.e. the hydrodynamic radius was larger. We speculate that this might be due to the bulky acyl chains of the galactolipids requiring more space. This effect was more pronounced for MGDG bicelles. MGDG and DGDG only differ in head group composition and thus size. The difference in hydrodynamic radius must thus be due to the different head groups. While DGDG is a bilayer-forming lipid, MGDG is not. It is likely that the additional curvature stress imposed on the bilayer by MGDG changes the morphology of bicelles and thus the hydrodynamic radius. Another explanation might be that the rim detergent DHPC mixes more efficiently with the bilayer of MGDG bicelles. Consequently, the effective q-value (ratio of lipids to detergents in the rim) may be higher, leading to an increase in hydrodynamic radius.

Bicelles that were produced from two E. coli strains (with and without PE) have somewhat larger diffusion coefficients than galactolipid bicelles but still fall in the typical range of small isotropic bicelles [102]. Both bicelle types have similar hydrodynamic radii.

We confirmed previous measurements of the lipid composition of the lipid extracts of both strains [4] by peak integration of $^{31}$P and $^{13}$C spectra. AD93WT has a typical E. coli lipid composition while AD93-PE does not synthesize PE and consists of 80% PG and 20% CL. The acyl chain composition depends on the growth phase and temperature. At 37 °C growth temperature the amount of double bonds substantially decreased and it increased at 20 °C. While the lipid head group composition of E. coli is typically insensitive to growth temperature [103], this is not the case for the AD93WT strain [104]. We found that PE levels decreased substantially when cells were grown at 37 °C while CL levels increased. Moreover, we observe that in the stationary phase the amount of cyclopropane moieties increased and CL is formed at the expense of PG. These observations are in accordance with previous studies [11, 105, 106]. Taken together, the lipid composition depends on growth phase, growth temperature, and strain. Therefore, the lipid composition of the bicelles is, to some extend, tunable and can thus be adapted to specific research questions.

Fig. 4.1 depicts the dynamic profile of lipids in bicelles made from E. coli lipid extract. The glycerol backbone displays high local order parameters ($S_{loc}^2$), i.e. it is rigid on a ps – ns timescale, while the acyl chain is highly dynamic and flexibility increases along the chain. This trend is interrupted by acyl chain modifications, like double bonds and cyclopropane moieties. The head group dynamics depend on the type of head group. For PC, PE, and PG $S_{loc}^2$ is similar to carbons in the acyl chain, i.e. small head groups are rather flexible. In contrast, CL (Fig. 4.1) and galactolipid (Fig. 4.2) head groups are rigid with $S_{loc}^2$ similar to that of the glycerols.
Figure 4.1: $S_{loc}^2$ of bicelles made from lipids purified from green: WT *E. coli* strain (AD93WT) and blue: PE-deficient strain (AD93-PE).

Figure 4.2: $S_{loc}^2$ of MGDG. The nomenclature of carbons in MGDG is depicted in the top panel.
4.2 Membrane interaction of the GT-B glycosyltransferase WaaG

The monotopic GT-B glycosyltransferase WaaG is involved in the synthesis of lipopolysaccharides (LPS) in Gram-negative bacteria. It adds the first outer core D-glucose to the inner-core L-glycero-D-manno heptose II of the nascent LPS chain. WaaG is located on the cytosolic side of the bacterial inner membrane and WaaG-deficient bacteria are non-motile and hypersensitive to novobiocin [107–109]. Therefore, WaaG has been identified as a potential antibiotic target [110].

The characteristic anionic charge of *E. coli* inner membranes was mimicked by increasing ratios of anionic lipids (POPG) substituting POPC in LUVs (0 – 40%) and by LUVs that mimicked the inner membrane composition of *E. coli*. The membrane-association of MIR-WaaG to LUVs was monitored by CD and fluorescence methods.

Upon increase of anionic charge α-helical structure was induced into MIR-WaaG and the fluorescence anisotropy increased indicating that the peptide binds electrostatically to membranes. Also, upon increasing the anionic charge the quenching efficiency by the soluble quencher acrylamide decreased while the quenching efficiency by nitroxide labeled lipids increased. Consistently, the parallax method showed that Tyr residues are on average located 16 Å away from the bicelle center, i.e. near the glycerol backbone of the lipids at the membrane-water interface. The dissociation constant of this interaction was 35 µM for LUVs mimicking the inner membrane composition of *E. coli*.

We then solved the NMR structure of the peptide in dodecylphosphocholine (DPC) micelles and found that it matched remarkably well with the corresponding sequence in the crystal structure. Relaxation enhancement studies conducted in the presence of Gd(DTPA-BMA) provided a detailed picture of residue localization in the micelle. The N- and C-terminus are solvent-exposed while a central part of the peptide (Y13 – T16) is located in a hydrophobic environment. Y19 – A23 are located on the surface. In agreement with fluorescence studies CLEANEX experiments showed NOE peaks between Tyr sidechains and water. Moreover, guadinium protons of all Arg residues exchanged with water. Using these results as qualitative constraints, the localization of MIR-WaaG could be modeled as shown in Fig. 4.3 where Tyr and Arg sidechains extend towards the interface of the micelle, the N- and C-terminus are located outside, and a central part of the peptide inside the micelle.

Combining the results of this study, we propose a model of the membrane interaction of WaaG in which MIR-WaaG anchors the protein to the membrane (4.4). Electrostatic interactions play a key role in membrane-association. Moreover, surface exposed Tyr residues as well as hydrophobic residues may also be involved in binding.

It has previously been found that the monotopic GT-B glycosyltransferases AtDG2 and A7MGS bind to anionic membranes through electrostatic and hydrophobic interactions [40, 62, 111, 112]. Moreover, A7MGS associates with one leaflet of anionic bilayers [63]. These observations gave rise to the model discussed in section 2.3 and our observations are in agreement with this model. However, they extend it slightly in that Trp residues are not necessarily required for membrane anchoring of MIR-WaaG. Instead, Tyr residues are found at the membrane interface and may fulfill the same role as Trp residues.
Figure 4.3: Localization of MIR-WaaG in DPC micelles. The NMR structure of the lowest-energy conformer is shown. Arg residues are in blue and Tyr residues in green. The micelle was generated with CHARMM [48–52].

Figure 4.4: Model of WaaG (PDB: 2IW1) anchoring to E. coli inner membranes. MIR-WaaG is highlighted in orange. The membrane was generated with CHARMM [48–52] and consists of 75% PE, 20% PG, and 5% CL.
5 Conclusions and Perspectives

Small isotropic bicelles are versatile membrane mimetics which are sufficiently small to allow for high-resolution solution-state NMR studies while at the same time providing a lipid bilayer. In order to enhance the biological relevance of such bilayers, bicelles that mimic plant and bacterial membranes were characterized. It was shown that galactolipids as well as natural lipids extracted from *E. coli* inner membranes can be incorporated into bicelles. The lipids in these bicelles exhibit significant dynamics, ranging from a relatively rigid glycerol backbone to highly mobile methyl groups.

The membrane interaction of the glycosyltransferase WaaG was characterized by identifying a membrane anchoring region of WaaG. Membrane anchoring is conferred by electrostatic interactions between the negatively charged bilayer and positively charged side chains. Additionally, Tyr and hydrophobic residues might play an important role in binding WaaG to the cytosolic leaflet of the inner membrane in *E. coli*.

In this work, lipid dynamics were studied in the absence of membrane proteins and NMR studies of a WaaG membrane anchor were conducted in micelles. An integration of both approaches can lead to detailed insights into membrane-lipid interactions. It is reasonable to assume that the binding of a peripheral membrane protein affects lipid dynamics. Specific interactions between lipids and proteins can, on the other hand, only be observed if the membrane mimetic system contains biologically relevant lipids. Moreover, affinities to membranes and dynamics of peripheral and monotopic glycosyltransferases are largely unknown. To resolve these questions, the study of plant and bacterial glycosyltransferases in membrane mimetics that faithfully mimic natural membranes is a promising approach.
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— It takes two to tango.

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— On second thoughts, it takes at least thirty-five to tango.
7 References


