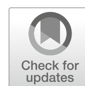

Solid-State NMR for Studying Peptide Structures and Peptide-Lipid Interactions in Membranes

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Erik Strandberg and Anne S. Ulrich

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Abstract

Peptide-lipid interactions can be conveniently studied using solid-state NMR (SSNMR), as various approaches have been developed to resolve the structures of membrane-bound peptides under quasi-native conditions. By labeling peptides with NMR-active nuclei, it is possible to characterize their conformation, orientation, and dynamics within a lipid bilayer and to obtain information about their self-assembly and aggregation behavior. This review is focused on peptides that are labeled with ^{2}H or ^{15}N and describe results primarily from two important classes of helical peptides: (i) hydrophobic transmembrane model peptides and (ii) amphipathic antimicrobial peptides. It can be concluded from these SSNMR studies that both types of peptides exhibit specific effects under conditions of

E. Strandberg (✉)
Institute of Biological Interfaces (IBG-2), Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany
e-mail: erik.strandberg@kit.edu

A. S. Ulrich
Institute of Biological Interfaces (IBG-2), Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

Institute of Organic Chemistry, KIT, Karlsruhe, Germany
e-mail: anne.ulrich@kit.edu

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hydrophobic mismatch, i.e., when the (hydrophobic) length of the peptide differs from the hydrophobic thickness of the bilayer. In particular, when the peptide is too long, it compensates this mismatch by tilting in the membrane, thereby providing an effective (hydrophobic) length to match the membrane thickness. It was also observed that peptides can more easily insert into membranes when the bilayer is composed of lipids with a large positive spontaneous curvature, such as lysolipids.

Keywords
Solid-state NMR · ^{2}H- and ^{15}N-labeled peptides · Alpha-helical membrane-bound peptides · Transmembrane model peptides · Amphipathic antimicrobial peptides · Peptide orientation and dynamics · Hydrophobic mismatch · Lipid spontaneous curvature · Geometric analysis of labeled alanine (GALA) method

Introduction

Solid-state NMR (SSNMR) is a powerful method for studying peptide-lipid interactions under quasi-native conditions. ^{31}P-NMR (from phospholipid head groups) and ^{2}H-NMR (from deuterated lipid acyl chains) can provide information on the response of the lipids when peptides are added to membranes. Likewise, ^{2}H-, ^{13}C-, ^{15}N-, and ^{19}F-labels can be selectively incorporated in the peptides, such that solid-state NMR experiments provide direct information on their conformation, orientation, and mobility within the membrane. This chapter will mainly discuss studies of labeled peptides in membranes. Another ▶ Chap. 32, "Solid-State ^{19}F-NMR Analysis of Peptides in Oriented Biomembranes," will be focused specifically on ^{19}F-NMR; therefore, this chapter will concentrate on the other nuclei as labels.

The most commonly used structural approaches are ^{15}N-NMR on backbone amide-labeled peptides and ^{2}H-NMR on Ala-d$_3$-labeled peptides carrying a CD_3-group on the backbone. A single ^{15}N label can be conveniently used to estimate the tilt angle of an α-helix from the chemical shift of the label [1]. Peptides can also be uniformly labeled with ^{15}N, and by performing a PISEMA-type 2D experiment [2], the tilt angle of an α-helical peptide can be resolved more accurately from the resulting PISA wheel [3, 4]. Amino acid type-selective ^{15}N labeling can then be used to determine also the azimuthal angle, which describes the rotational angle around the helix axis and is of particular importance when studying amphipathic peptides [5]. Ala-d$_3$-labeled peptides are most readily examined using the geometric analysis of labeled alanine (GALA) method, where several positions are labeled one by one, as in an alanine scan. The splittings of these positions are analyzed in terms of helical waves to determine the overall peptide orientation (tilt angle and azimuthal rotation) and the dynamics [6, 7]. ^{13}C-NMR of selectively labeled peptides has also been applied in some studies [8–10].

Natural biological membranes are very complex and are tedious to prepare from cells. Therefore, model membranes composed of synthetic lipids or lipid mixtures are used in most biophysical studies. Only few SSNMR studies have focused on native membranes that were prepared from microorganisms, and the results obtained

were similar to those from model systems [11, 12]. Model membranes can be prepared with different properties, as a wide range of lipids with different head groups, chain lengths, and chain saturations, as well as lysolipids and branched lipids, are commercially available and have been used in SSNMR studies [13–15]. This approach makes it possible to systematically investigate the influence of different lipid properties on membrane-bound peptides.

It is often advantageous to use oriented samples in SSNMR studies. NMR parameters, such as dipole-dipole couplings or quadrupolar couplings, are orientation dependent; therefore, an unoriented sample, which includes all orientations of molecules, produces broad spectral lines. On the other hand, in an oriented sample where all peptides have the same orientation with respect to the static magnetic field direction, sharp lines are obtained, and the signal-to-noise ratio is dramatically improved. One approach that is used to obtain oriented membrane samples makes use of bicelles, which are small disc-shaped membrane fragments that usually consist of a mixture of two components, one covering the rim of the disc and one forming the bilayer in the center [16, 17]. Such bicelles tend to align themselves in a magnetic field with the membrane director perpendicular to the magnetic field, but they can also be flipped by 90° by the addition of paramagnetic ions [16, 18]. Often, such bicelles are stable at only certain temperatures and lipid compositions. A more robust method for sample preparation is to orient membranes macroscopically on thin glass plates [7, 19–21]. This approach produces extended flat bilayers that can be tilted at any angle with respect to the magnetic field using a goniometer NMR probe. Virtually any lipid composition and peptide-to-lipid molar ratio (P/L) can be used, as long as it is possible to obtain a good orientation of the peptides and detect them at low concentrations.

Non-oriented multilamellar vesicles (MLVs) have been used in some studies. If the peptide is motionally averaged around the bilayer normal on an NMR time scale, then such a vesicle sample will produce splittings that can be analyzed in a manner similar to those from an oriented sample [6, 22]. One advantage of using MLV samples is that larger amounts of labeled peptides can be used; in oriented samples, much of the sample volume is filled with the glass plates. On the other hand, in MLV samples, an unbinding and escape of water-soluble peptides from the membrane may be an issue [23]. In oriented samples, the water content is usually low (close to saturation); hence peptides are forced to bind to the membrane. In MLV samples, however, there is an excess of free water, and peptides with a low binding affinity will give a predominantly isotropic NMR signal. In such cases, electrostatic attraction between the peptides and the membrane may be utilized for effective binding, i.e., for cationic peptides, it is advisable to include anionic lipids in the lipid bilayer [23].

Systems Studied

SSNMR is a well-established method that has been used in many studies of peptides in membranes. It is not possible to discuss all of these studies here; however, papers on antimicrobial peptides (AMPs) have been listed in previous reviews [13, 14].

A choice has been made here to review studies in which SSNMR was used to obtain a better understanding of peptide-lipid interactions, in particular with regard to peptide length, bilayer thickness, and spontaneous membrane curvature. These results provide insights into the fundamental factors that are involved in peptide-lipid interactions and can thus assist with selecting suitable sample conditions for future studies.

Model Transmembrane Peptides

The WALP series of peptides are hydrophobic model sequences with different lengths that align in a transmembrane fashion, resembling membrane-spanning protein segments. They were designed to study the effects of hydrophobic mismatch [24], which occurs when the hydrophobic length of the peptide differs from the hydrophobic thickness of the lipid bilayer. These peptides have a central (Leu-Ala)$_n$ stretch that is flanked on either side by two Trp residues, and the charges on both termini are capped. The general sequence is acetyl-GWW(LA)$_n$LWWA-amide (and its total length x is given in the name WAPx). Using ^{31}P-NMR to study non-oriented membranes and upon varying either the peptide length or the membrane thickness, it was shown that a negative mismatch (where the peptide is too short to span the hydrophobic thickness of the membrane) can induce non-lamellar isotropic or hexagonal lipid phases [24–26] in a mismatch-dependent manner. Similarly, peptides that resemble WALP but have other types of aromatic, charged, or polar flanking residues in place of Trp were also found to induce non-lamellar phases. From these studies, the preference of different amino acids to localize at the lipid-water interface could be examined [27, 28].

Ala residues in the hydrophobic core of WALP peptides were labeled with Ala-d$_3$, and from ^{2}H-NMR studies on these peptides, the orientation of peptides in the membrane could be directly determined [6, 7, 29]. Using WALP19, WALP23, and four different lipid systems with different hydrophobic thicknesses (DmPC, with m = 12:0, 13:0, 14:0, 18:1), it was found that the peptides could readily adapt to a positive mismatch – where the peptide is too long – by tilting, enabling the helix to assume a shorter effective hydrophobic length in the membrane (see Fig. 1). However, it turned out that peptide dynamics must be properly taken into account in the analysis of the NMR data to obtain reliable and quantitative results [29–31].

A similar mismatch-dependent tilt was also observed in ^{15}N-NMR studies of the viral peptide Vpu [32]. For backbone ^{15}N-labeled helices, there is also an influence by the dynamics, but this tends to be less critical than for side-chain ^{2}H-labels, due to the different alignment of the NMR tensors with respect to the helix axis [31, 33, 34]. Mismatch effects have also been observed by ^{15}N-NMR for the transmembrane parts of the oncoprotein E5 and the PDGF-receptor, which interact with one another to form an activated signal-transduction complex [35–37].

A series of WALP-like peptides with only one Trp at each terminus was shown to be easier to handle and less dynamic than WALPs, so these peptides have been used in further ^{2}H-NMR studies. They are called GWALP [38] or, more generally, XWALP [39], where X is an outer residue and W is an inner flanking residue at

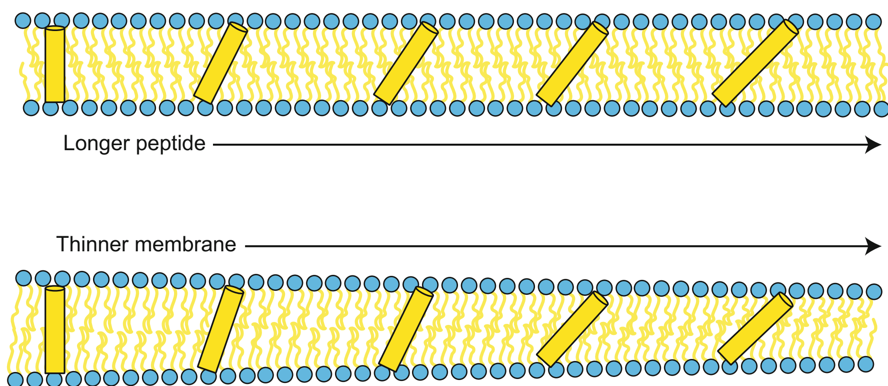

Fig. 1 Transmembrane helices, such as WALP peptides (*yellow cylinders*), adapt their tilt angle so that their hydrophobic stretch fits into the hydrophobic interior of the membrane by minimizing the hydrophobic mismatch. Lipids are depicted with polar head groups in *blue* and hydrophobic acyl chains in *yellow*

each end, with the sequence acetyl-GXALW(LA)$_6$LWLAXA-amide [40–42]. Using these peptides, the influence of different flanking residues, such as Trp, Tyr, or Phe, was examined. In addition, the effect of special amino acids placed into the hydrophobic region, such as charged residues [43, 44] or helix breakers, was also investigated [45].

The WALP and GWALP peptide studies primarily used ^{2}H-NMR on Ala-d$_3$-labeled peptides. However, in some cases, ^{15}N-labels were also introduced into the peptides, and both methods have been compared in the same peptide-lipid system and shown to yield identical results [33, 38].

Amphipathic α-Helical Peptides

Among the most prominent amphipathic α-helical systems are AMPs, which can kill microorganisms by permeabilizing the cellular membranes. Because of their potential pharmaceutical use, these peptides have been intensely studied using SSNMR methods. Due to their regular secondary structure, the α-helical ones are ideally suited for these studies, because one or a few labels are sufficient to determine the orientation of the peptide in the membrane. The amphiphilic helices are mostly seen to lie on the bilayer surface, which may contribute to membrane damage via bilayer thinning or other indirect mechanisms. However, if a helix is found to have a transmembrane orientation, this alignment is a clear indication that several monomers must have assembled into a stable pore or slit that permeabilizes the membrane.

PGLa is a 21-amino acid AMP from the African frog *Xenopus laevis* and represents one of the most studied systems using SSNMR. Several highly sensitive ^{19}F-NMR structure analyses of PGLa in various types of membranes under many different conditions are described in ▶ Chap. 32, "Solid-State ^{19}F-NMR Analysis of

Peptides in Oriented Biomembranes." An early ^{15}N-NMR study in POPC/POPG membranes used many labeled positions to demonstrate that PGLa has a flat orientation on the membrane surface [46]. Subsequent ^{2}H-NMR experiments demonstrated a concentration-dependent helix flip in DMPC bilayers into a tilted orientation at higher P/L ratios [22, 23]. This realignment was not observed in POPC, however, where the helix always remained flat on the surface, even at very high P/L ratios. This ^{15}N-NMR study thus showed that the ability of PGLa to reorient is clearly lipid dependent [47]. Using ^{19}F-NMR, it was furthermore found that the peptide assumes a flat surface-bound state in DMPC at elevated temperature, while at ambient temperature, it becomes tilted, and at even lower temperatures – close to the liquid-to-gel-phase transition temperature – it assumes a transmembrane orientation [48]. An overview of the factors affecting peptide orientation is summarized in Fig. 2.

Magainin 2 (MAG2) is a related AMP found in the skin of *X. laevis* along with PGLa. The orientation of MAG2 in POPC was determined using ^{15}N-^{1}H PISEMA [49], which revealed a flat orientation on the membrane surface. A recent study using ^{2}H-NMR and molecular dynamics simulations confirmed that MAG2 is essentially always flat in both POPC and DMPC [50]. Of special interest is the strong synergistic activity found for a combination of PGLa/magainin, as reviewed in [51]. A 1:1 mixture of these peptides leads to much better killing of bacteria, induces greater

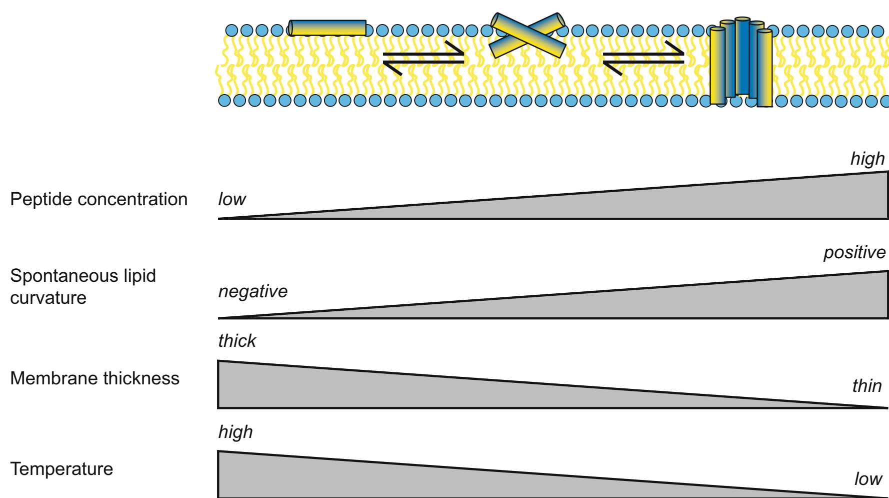

Fig. 2 Factors influencing the insertion of amphipathic peptides into membranes. A surface-bound peptide can realign in a lipid bilayer via a tilted state (presumably a dimer) and further into a fully inserted transmembrane orientation (presumably an oligomeric pore). For typical α-helical peptides with lengths of around 20 amino acids, this stepwise equilibrium has been found to be promoted by high peptide concentrations, thin membranes, positive spontaneous curvatures, and low temperature (lipid gel phase). Lipids are depicted with polar head groups in *blue* and hydrophobic acyl chains in *yellow*. Peptides are shown as cylinders with the polar region in *blue* and the hydrophobic region in *yellow*

vesicle leakage, and causes stronger membranolytic effects than expected from the sum of the activities of the two peptides. Notably, ^{2}H-NMR demonstrated that PGLa assumes a transmembrane state in DMPC/DMPG bilayers in the presence of MAG2 [51, 52], which suggests the formation of stable pores made up of heterodimeric peptide complexes. However, in the same system, MAG2 was not found to be inserted in the presence of PGLa [53, 54], leading to the model in which PGLa lines the inside of a water-filled pore, while MAG2 lies on the membrane surface and stabilizes the pore through some interaction with PGLa. In contrast, in POPC bilayers, neither PGLa was inserted in the presence of MAG2 nor was MAG2, indicating that peptide-lipid interactions are also important for the formation and stability of the pores [53–55].

Additional insight into this peptide-lipid interplay was provided by ^{2}H-NMR studies of MSI-103, a designed AMP with the sequence (KIAGKIA)$_3$-NH$_2$, which is based on the sequence of PGLa but is more active [56]. This amphipathic α-helical peptide was first studied in DMPC [57] and behaves much like PGLa, with a concentration-dependent realignment and with almost exactly the same orientational states. The peptide orientation was then examined systematically in a wide range of lipid systems [58], and it was noted that in some lipids, such as DMPC and DLPC, MSI-103 became tilted at a high P/L ratio, whereas in other lipids, such as POPC or DOPC, a flat orientation was always observed. By including further lipids with different head groups as well as lysolipids in the membranes, it could be concluded that there is a strong correlation between the intrinsic spontaneous curvature of the lipids and the tendency of the peptide to undergo reorientation [53, 58]. In lipids with a positive spontaneous curvature, such as DMPC, the helix was found to insert more readily in a transmembrane state than in lipids with a negative curvature, such as POPC. The effect is most pronounced in lipids with an extreme positive curvature, such as lyso-PC, or an extreme negative curvature, such as PE (Fig. 2). Recently, it was demonstrated that the effect of spontaneous curvature on helix reorientation and on the stability of a transmembrane state is not specific to the α-helical peptides mentioned here. Rather, it is a general effect also for other antimicrobial peptides, such as the amphipathic gramicidin S with a cyclic backbone [59], and even for large β-barrel proteins, such as porins from the outer membranes of bacteria [13].

To understand and resolve the differential influence of spontaneous lipid curvature and of bilayer thickness, a series of peptide analogues was designed with different lengths. Based on a repeated [KIAGKIA] motif from the MSI-103 sequence, a set of nine so-called KIA peptides were prepared with lengths from 14 to 28 amino acids [60]. When comparing their membranolytic activities, it was found that only helices that are long enough to span the membrane could induce vesicle leakage, indicating that any peptides that were too short remained inactive because they could not form pores [60]. The KIA peptides were also characterized using ^{15}N-NMR in several different lipid systems. As seen with MSI-103, they were only able to insert in a transmembrane state when the lipids had a positive spontaneous curvature. In POPC and in DErPC, which have unsaturated acyl chains and a negative curvature, all KIA peptides were found to remain on the membrane surface, but a transmembrane orientation was observed in DMPC/lyso-MPC [61]. Using

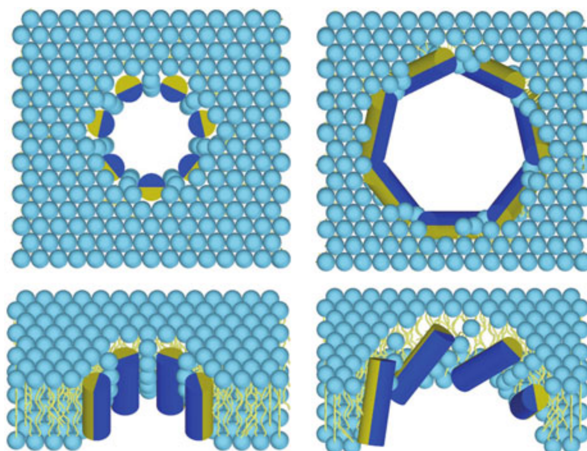

Fig. 3 When amphipathic helices, such as the KIA peptides, are inserted in a transmembrane state, they adapt their tilt angle to the membrane thickness. The *upper panels* show the resulting putative pores from the top, and the *lower panels* show a section through the pores. On the *left*, the peptide length matches the hydrophobic thickness of the membrane, and the pore is formed by upright peptides. On the *right*, the peptides are longer and become tilted to match the membrane thickness, thus forming a skewed pore. Adapted from Grau-Campistany et al. [61]

such lysolipid-containing system, it was possible to establish ideal conditions under which the KIA peptides are homogeneously trapped in a transmembrane state. Since they have one charged and one hydrophobic face, they cannot span the bilayer as monomers, as this would expose charged residues to the hydrophobic interior of the membrane. Therefore, they are probably arranged as an oligomeric pore so that the charged groups are lining a water filled center [61]. SSNMR structure analysis of these complexes showed that the tilt angle of the KIA peptides depends on the peptide-lipid mismatch, i.e., the shortest helices are aligned essentially upright in the membrane, whereas the longer ones become progressively tilted. Notably, the tilt angle varies smoothly with the membrane thickness, with peptides becoming more tilted in thinner membranes (Fig. 3). This result indicates that – upon pore formation – the charged, amphipathic KIA peptides adapt their tilt angles to the membrane according to the concept of hydrophobic matching, in a manner similar to that described above for the fully hydrophobic WALP peptides (Fig. 1). This behavior had not been observed previously but can now be investigated under the newly found conditions promoting the pore state [61].

In contrast to the KIA peptides, which exhibit signs of pore formation with a minimal length of at least 17 amino acids, other designated AMPs are clearly too short to span a membrane. The amphiphilic helix of BP100 consists of only 11 amino acids, yet it possesses, both, a high antimicrobial activity toward bacteria and an excellent cell-penetrating efficiency toward eukaryotic cells [62, 63]. The orientation of BP100 in terms of its tilt angle and rotation angle could be resolved by a combination of ^{19}F-, ^{15}N-, and ^{2}H-NMR, as well as oriented CD data

[64–66]. The compact peptide was observed to remain on the membrane surface with a high mobility and was therefore proposed to act via a carpet mechanism.

Conclusions

SSNMR has been developed into a routine method to examine the orientation and dynamics of α-helical peptides in membranes. Systematic studies of different types of peptides in a variety of lipid systems have yielded quantitative insights into functionally relevant peptide-lipid interactions. In particular, the hydrophobic mismatch-dependent tilting that had been predicted by theory has been confirmed, not only for fully hydrophobic peptides but also for amphipathic peptides that are arranged as transmembrane pores. Furthermore, it has been demonstrated that lipids with a positive spontaneous curvature promote the insertion of peptides into membranes, not only in the case of amphiphilic α-helices but even of larger β-pleated proteins. While lipid curvature plays the most critical role of allowing the insertion of an amphiphilic helix as such, membrane thickness then serves as a secondary factor to modulate the effective tilt angle of the inserted segment.

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