^{1}H High-Resolution Magic Angle Spinning NMR Spectroscopy for the Investigation of a Ras Lipopeptide in a Lipid Membrane**

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Membrane association is a major prerequisite for biological function of different proteins involved in cell signaling such as trimeric G proteins or the oncogen *Ras*. A salient feature of these proteins is that membrane affinity and binding is achieved by co- or posttranslational lipidation of amino acids located near the N or C terminus of the protein.[1] Covalent attachment of myristic or palmitic acid and prenyl chains equip proteins with a hydrophobic membrane anchor directing them towards the cell membrane.

Currently, structural data on membrane-bound lipid-modified peptides are not available mostly because of missing synthesis pathways for lipidated model compounds and the failure of standard techniques for structural investigations such as X-ray crystallography and solution NMR spectroscopy to resolve structural information in the membrane environment. Recently, however, lipid-modified peptides were synthesized.[2] These peptides not only provide excellent model systems for structural studies but also a new efficient route to lipid-modified proteins, which show activity almost as high as that of the wildtype protein.[3]

For soluble peptides, ^{1}H NMR spectroscopy is a standard method to obtain high-resolution molecular structures. However, this method fails for membrane-bound peptides because these molecules do not undergo fast isotropic tumbling on the NMR time scale. Consequently, ^{1}H NMR lines are homogeneously broadened (typically by several kHz) due to strong anisotropic ^{1}H–^{1}H dipolar couplings, resulting in broad featureless line shapes.

For pure lipid membranes, solution-like ^{1}H NMR spectra can be obtained if the sample is spun about an axis oriented at 54.7° with respect to the external magnetic field (the "magic angle"; magic angle spinning, MAS). Significant resolution enhancement is achieved even at spinning speeds not exceeding the effective dipolar couplings due to fast axially symmetric motions of the lipids in the membrane.[4] Membrane-bound peptides, however, undergo only slow motions that broaden NMR lines and restrict spectral resolution. Recent ^{1}H high-resolution magic angle spinning (HR MAS) NMR investigations on membrane-bound peptides at very high spinning speeds (ca. 15 kHz) have partially overcome these limitations.[4b, 5] Nevertheless, according to Davis et al. "to observe the high-resolution ^{1}H NMR spectra of either peptides or lipids in an ordered system such as a model or biological membrane, it is absolutely necessary to have rapid axially symmetric molecular reorientation".[4b]

Herein we present a ^{1}H HR MAS NMR study of a lipid-modified, membrane-bound heptapeptide, which models the C terminus of the human N-Ras protein. This method allows for the first time structural investigations of a lipid-modified, membrane-bound peptide. Membrane binding is achieved through a covalently bound palmitic fatty acid chain and a hexadecyl thioether, which are built into the membrane. This binding motif provides the peptide with motional freedom to perform axially symmetric reorientations in the bilayer, which improve the NMR line widths significantly. The amino acid sequence of the peptide investigated is H-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(HD)-OMe (Pal: palmitic acid, HD: hexadecyl). The peptide was incorporated into deuterated 1,2-dimyristol-*sn*-glycero-3-phosphocholine ([D_{67}]DMPC) multilamellar vesicles and investigated by ^{1}H HR MAS NMR spectroscopy.

Figure 1 displays the ^{1}H MAS NMR spectrum of the lipid/peptide dispersion. Besides the strong water peak at $\delta = 4.55$ the spectrum shows very intense signals from the fatty acid chains of the peptide as well as glycerol groups of the phospholipid due to incomplete deuteration of [D_{67}]DMPC.[6] Typical line widths of these phospholipid signals are 0.05–0.06 ppm (approximately 30 Hz on the 600 MHz instrument). Furthermore, a large number of aliphatic peptide signals are well-resolved. The line width for these side chain signals is

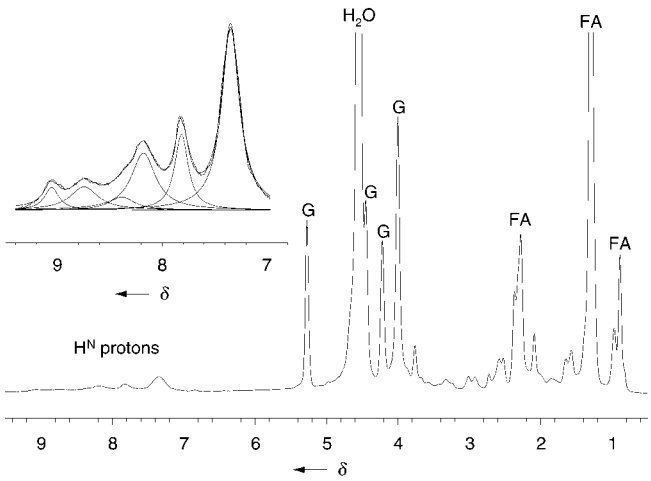

Figure 1. ^{1}H HR MAS NMR spectrum of the Ras peptide in [D_{67}]DMPC multilamellar liposomes (30 wt % H_2O) at a spinning speed of 12 kHz and a temperature of 37 °C. Peaks labeled with G represent glycerol signals of the phospholipid, which are not ^{2}H labeled in [D_{67}]DMPC.[6] Fatty acid signals (FA) of the peptide acyl chains and a large number of well-resolved aliphatic peptide signals are observed in the upfield region of the spectrum. The inset shows the amide region of the spectrum; the line shape of the amide signals could be fit with six Lorentzian lines.

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only slightly broader than those for the lipids (0.07 – 0.1 ppm). In the downfield region of the spectrum, the six amide protons of the peptide are resolved (proline has no amide proton). This spectral region could be fit by using six Lorentzian lines with a line width of between 0.16 and 0.32 ppm (see inset of Figure 1). The H^N proton signals are broadened by solvent exchange and scalar relaxation with the neighboring ^{14}N nuclei. The peptide backbone also represents the most rigid region of the peptide, and line broadening due to residual dipolar couplings is particularly effective. The intensity differences observed for the H^N signals could be due to the varying mobility of the amide bonds in the peptide backbone, as more mobile structures have more centerband and less sideband intensity because of weaker effective dipolar couplings. In contrast, more rigid structures exhibit stronger dipolar couplings and, consequently, more sideband and less centerband intensity.[6]

Increased resolution and long spin-lattice relaxation times allow acquisition of 2D nuclear Overhauser enhancement spectra (NOESY). In this way magnetization exchange of protons can be probed up to distances of 5 Å.[7] For HR MAS studies, the same NOESY pulse sequence as used in high-resolution NMR spectroscopy is applied. Magnetization transfer is monitored by the appearance of a non-diagonal cross-peak in the 2D NOESY spectrum. Figure 2 shows the

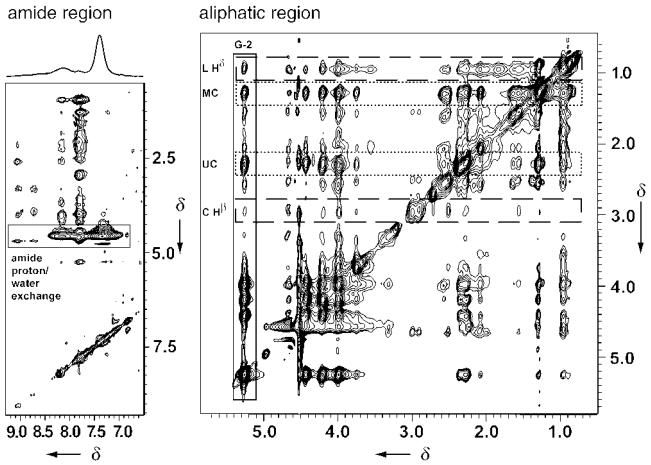

Figure 2. ^1H HR MAS NOESY spectrum of the Ras peptide in a $[D_{67}]$DMPC multilamellar liposomes at a mixing time of 200 ms. The aliphatic region shows cross-peaks of the glycerol (G-2) signal with various peptide and lipid signals (solid box). Dashed boxes emphasize cross-peaks for the β protons of the Cys residues ($C H^{\beta}$) and the δ protons of the leucine residue ($L H^{\delta}$), respectively. Cross-peaks of the upper (UC) and middle (MC) peptide's acyl chains are shown in the dotted box. In the amide proton region cross-peaks due to exchange of labile protons with the solvent are highlighted. A section of the 2D spectrum taken at the shift of the water signal ($\delta = 4.55$) is also displayed, which confirms that all amide protons participate in the exchange with water.

aliphatic and H^N region of the ^1H HR MAS NOESY spectrum at a mixing time of 200 ms. Typically, three types of cross-peaks are observed. a) Intramolecular cross-peaks due to magnetization transfer within the peptide. This magnetization transfer is modulated by the overall motion of the molecule on the membrane surface and can be converted into proton – proton distance constraints for structure calculations. b) Intermolecular cross-relaxation between lipid and peptide is modulated by variations of the internuclear distance vector through motions of the entire molecules. Since intermolecular distances vary significantly in liquid crystals, these cross-relaxation rates cannot be converted into fixed molecular distances but rather represent the contact probability between molecular segments[6, 8] and are used for their location in the membrane.[9] c) Chemical exchange between labile H^N protons and water gives rise to cross-peaks that provide information on the water accessibility of amino acids in the membrane.[10]

In the NOESY spectra of membrane-bound Ras peptides all three types of cross-peaks are observed. It is beyond the scope of this communication to provide a full assignment and discussion of all occurring cross-peaks; however, a few interesting correlations are highlighted. Our tentative assignment of the signals is based on the assignment of the signals of the peptide in $CDCl_3$.[2] For instance, the H^{δ} protons of Leu at $\delta = 0.95$, show very strong cross-peaks with the glycerol and the upper chain region of the phospholipids. Similarly, the H^{β} protons of the two Cys residues ($\delta = 3.01$ and $\delta = 2.92$) show cross-peaks with glycerol and upper chain signals of the lipids; however, no contacts between the Cys-H^{β} protons and the methylene or methyl segments of the peptide acyl chains are observed. This suggests that the location of Leu and Cys side chains is in the glycerol and upper acyl chain region of the membrane. This is consistent with the assumption that insertion of the peptide's lipid chains in the membrane drag the Cys side chains deeper into the nonpolar lipid region of the membrane, allowing favorable interactions with upper acyl chain segments. According to the experimental hydrophobicity scale developed by Wimley and White[11] the total free energy of transferring the peptide from the aqueous phase to the membrane is -0.8 kcal mol^{-1}, indicating the high propensity of the Ras peptide for the membrane even in the absence of lipid modification.

To further probe the location of the peptide in the lipid bilayer the exchange of labile amide protons with water was analyzed. In the H^N region of the NOESY spectrum, intramolecular cross-peaks between amide protons and peptide side chains are observed as well as intermolecular cross-peaks with glycerol and acyl chain signals (Figure 2). In addition, the spectrum shows three strong cross-peaks due to the chemical exchange of H^N protons with H_2O, whereas the other amide protons show weak exchange cross-peaks. By evaluating the section of the 2D spectrum at $\delta = 4.55$, it can be verified that all H^N protons participate in the exchange with water. In contrast, amide protons, which are buried in the hydrophobic core of the membrane, are protected from water exchange, whereas amino acids located in the lipid/water interface are exchangeable.[12] For the Ras peptide, all amide protons participate in the water exchange, suggesting the location of the peptide backbone is in the water-accessible part of the membrane, that is in the lipid/water interface defined by the headgroup, the glycerol units, and the upper chain region.[13]

In summary, we have shown that the ^1H HR MAS NMR spectra of a membrane-bound lipopeptide provide excellent spectral resolution for the structural investigation of these model systems. The Ras peptide was located in the lipid/water

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interface of the membrane, while the peptide acyl chains are inserted into the interior of the membrane (Figure 3). The lipid/water interface of the membrane represents a broad zone containing disordered headgroups, glycerol, and upper chain segments, which is easily accessed by water molecules.[13]

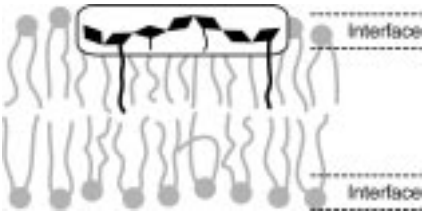

Figure 3. Schematic representation of the location of the Ras peptide in the lipid/water interface of the membrane (headgroup, glycerol, and upper chain region). The peptide acyl chains and hydrophobic side chains extend deeper into the hydrophobic part of the membrane.

The hydrophobic peptide side chains project into the acyl chain region of the membrane, where the low polarity environment allows favorable hydrophobic interactions with the lipid chains. The polar backbone of the peptide interacts strongly with the aqueous phase. For the peptide/lipid system investigated here a full assignment of the NMR signals as well as a quantitative analysis of the location and membrane-bound structure of the peptide appears to be feasible by using HR MAS NMR spectroscopy, thus providing more insight into this important mechanism for membrane binding and protein insertion.

Experimental Section

$[D_{67}]$1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine ($[D_{67}]$DMPC) was used without purification. The lipid-modified N-Ras peptide was synthesized enzymatically as described before.[2] For the preparation of the NMR samples, phospholipid and peptide (molar ratio of 10:1) were combined in methanol, dried under a stream of nitrogen, dissolved in cyclohexane and lyophilized. The samples were hydrated to 30 wt% H_2O, freeze-thawed, stirred, and gently centrifuged for equilibration and transferred into spherical inserts for 4-mm MAS rotors. NMR experiments were carried out on a Bruker DRX600 spectrometer at a resonance frequency of 600.13 MHz at 37 °C. The MAS frequency was 12 kHz. Spectra were acquired at a spectral width of 10.9 kHz with a 90° pulse length of 6.2 μs. For phase-sensitive NOESY experiments (mixing time 200 ms) 400 complex data points were collected in the indirect dimensions with 32 scans per increment with a 4 s relaxation delay between successive scans.

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Enantioselective Synthesis of the Ricciocarpins A and B**

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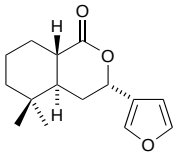
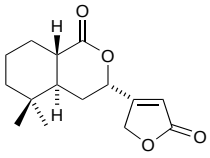
Dedicated to Professor Wittko Francke on the occasion of his 60th birthday

The furanosesquiterpene lactone ricciocarpin A (**1**) isolated from the liverwort *Ricciocarpos natans* exhibits high molluscicidal activity against the water snail *Biomphalaria glabrata*, one of the vectors of schistosomiasis (bilharziasis).[1] Though several syntheses were published for racemic **1**,[2]


ricciocarpin A (**1**)


ricciocarpin B (**2**)

no enantioselective version has so far been reported. Since the absolute configuration of **1** was unknown prior to our work, we principally wanted to devise an access to both enantiomers. Moreover, we were also interested in developing a first synthetic access to the structurally similar liverwort-constituent ricciocarpin B (**2**).[1] Our synthesis of **1** reported here relies heavily on the twofold application of the catalytic ring-closing metathesis (RCM)[3] to generate both six-membered rings of the target.

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