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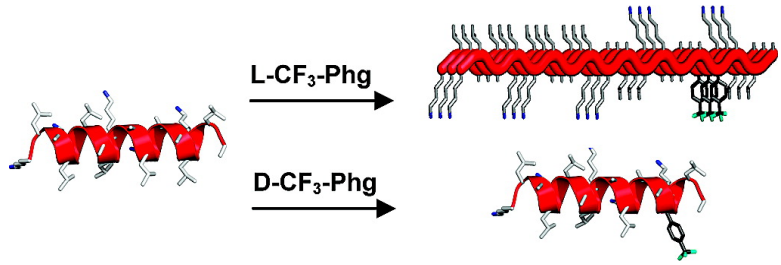

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Using a Sterically Restrictive Amino Acid as a ^{19}F NMR label To Monitor and To Control Peptide Aggregation in Membranes

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Spontaneous aggregation of peptides in the presence of membranes is often implicated as a crucial step in various amyloid-related disorders.[1−3] Many membrane-active peptides, such as cell-penetrating or fusogenic sequences, are also known to have a strong tendency to aggregate as β-sheet structures.[4,5] It is not clear, however, whether such aggregation is an intrinsic step in their mechanism of action, or whether it is a side-effect that should rather be avoided when designing a therapeutic peptide. The main obstacle toward answering such a functionally relevant question is not only the experimental difficulty of observing peptide aggregation in the membrane-bound state, but also the necessity to actively control the aggregation behavior of the system. Ideally, an aggregating peptide plus a nonaggregating analogue would be required for structural characterization and for comparative functional tests.

Here, we present a strategy to simultaneously control and monitor the aggregation behavior of peptides in the membrane-bound state. This approach makes use of a sterically restrictive ^{19}F-labeled amino acid, which can be observed by highly sensitive solid state ^{19}F NMR.[6−12] The key concept is to incorporate the rigid amino acid as either an *L*- or a *D*-enantiomer, in order to allow or to sterically prevent β-sheet aggregation, respectively.[6,13−15] Figure 1 illustrates that both the *L*- and *D*-forms of 4-CF_3-phenylglycine (CF_3−Phg) can be accommodated in the typical α-helical conformation of a membrane-active peptide. In contrast, the assembly of oligomeric β-strands will be prevented in the case of the *D*-form, as the configuration of the rigid side chain would disrupt the intrinsic hydrogen-bonding pattern of β-sheets. We may thus expect that the *D*-epimeric peptide cannot aggregate, while the *L*-epimer should have a similar aggregation tendency as the wild-type sequence, from which it is typically derived by substitution of a bulky hydrophobic amino acid such as Leu, Ile, or Phe.

To prove this concept, we have investigated here the model amphipathic peptide ("MAP", KLALKLALKALKAALKLA-amide), which had been designed as an amphiphilic α-helix by Steiner et al.[16,17] The cationic membrane-active peptide has pronounced antimicrobial and hemolytic activities. It has also been used as a cell-penetrating sequence that is able to efficiently traverse lipid bilayers and deliver covalently attached cargo into cells.[18] Its helical conformation was confirmed by circular dichroism (CD) in various membrane-mimicking and lipidic environments. At high concentration, however, CD and infrared reflection absorption spectroscopy (IRRAS) showed that MAP can aggregate as β-sheets, as promoted by the 2D bilayer plane.[19]

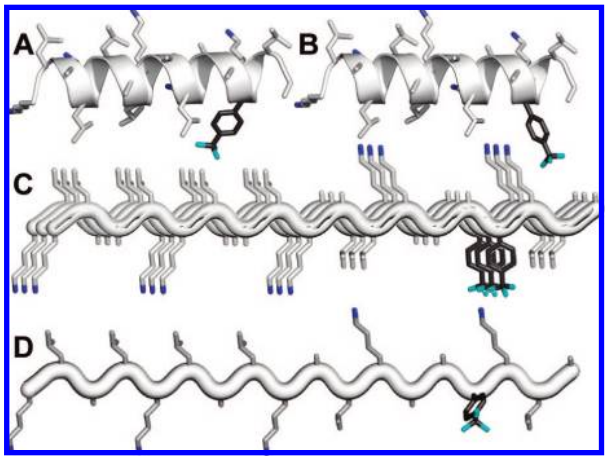

Figure 1. An α-helical peptide (here, MAP) can accommodate both (A) *L*-CF_3−Phg and (B) *D*-CF_3−Phg, whereas an oligomeric β-sheet is compatible only with the *L*- but not the *D*-form of the amino acid (C, D), hence aggregation of the *D*-epimeric peptide is sterically hindered.

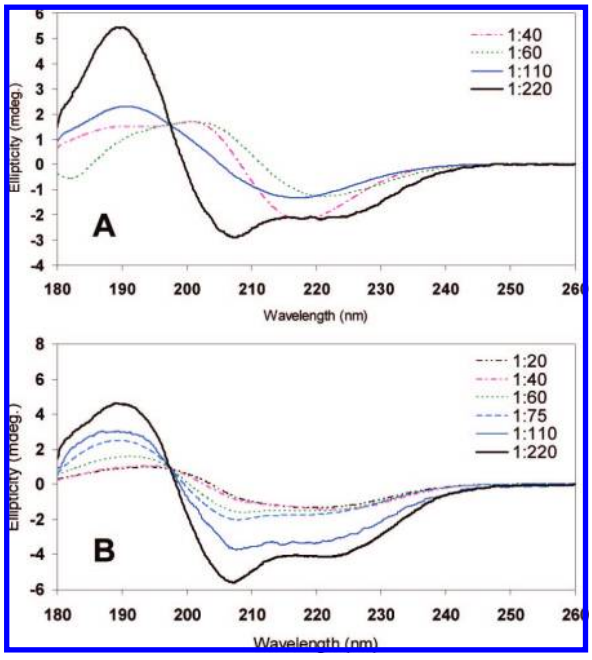

Figure 2. Oriented CD spectra of MAP labeled with (A) *L*-CF_3−Phg and (B) *D*-CF_3−Phg, at different peptide-to-lipid ratios in DMPC at 37 °C.

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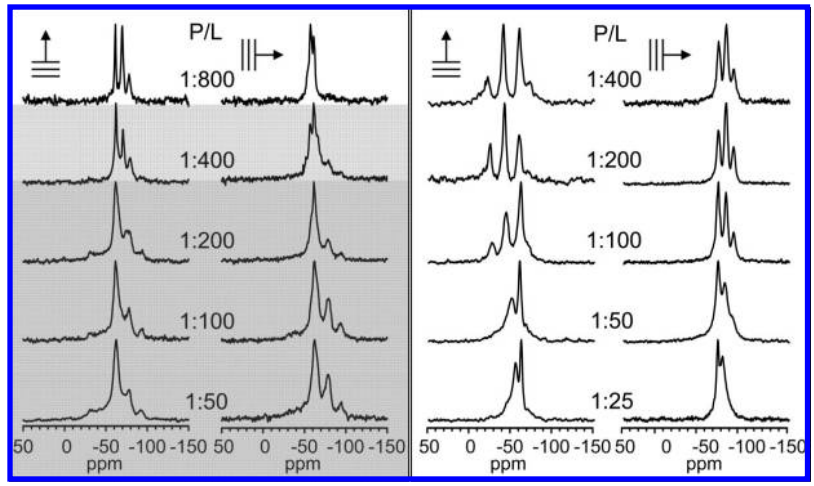

Figure 3. Solid state ^{19}F NMR spectra of MAP epimers labeled with *L*-CF_3−Phg (left panel) and *D*-CF_3−Phg (right panel), in DMPC at different peptide-to-lipid ratios (P/L). The spectra were measured in the liquid crystalline state of DMPC with the sample normal aligned parallel (0°,↑) and perpendicular (90°, →) to the static magnetic field.

To control peptide aggregation and to simultaneously detect the resulting loss in mobility by ^{19}F NMR, we have specifically labeled MAP with a single *L*- or *D*-CF_3−Phg substituent in the position of Leu6, Leu8, Leu11, or Leu15. First, the overall conformation of these analogues was monitored by CD (see Supporting Information) and by oriented CD (OCD) in macroscopically aligned DMPC bilayers at different peptide-to-lipid ratios (P/L, mol/mol). Figure 2B proves that the *D*-epimeric MAP peptide (labeled here at Leu15) indeed assumes an α-helical conformation at all concentrations, as expected. Note that the characteristic change in the OCD line shape at 1:40 represents a realignment of the helix from a surface-bound S-state to an obliquely tilted T-state in the lipid bilayer, as previously demonstrated for related amphiphilic peptides.[20] The corresponding *L*-epimer of MAP (Figure 2A), on the other hand, can only maintain its helical conformation below P/L of 200 before its starts to aggregate at higher peptide concentration. The alignment of the β-strands with respect to the bilayer cannot be extracted, but most likely they lie parallel to the surface as suggested by IRRAS at the air−water interface.[19]

Next, using the same kind of oriented DMPC samples as for OCD, solid state ^{19}F NMR was used to obtain more detailed information in the structure and mobility of the membrane-bound MAP peptide. We determined the angle of each CF_3-labeled side chain with respect to the membrane normal, using simple ^{1}H-decoupled 1-pulse ^{19}F NMR experiments as previously described.[9,21−26] We also used ^{31}P NMR to demonstrate that the lipid bilayer remains largely unperturbed (see Supporting Information). Figure 3 shows representative ^{19}F NMR spectra for the *L*- and *D*-epimers of one of the MAP analogues (labeled here at Leu15) as a function of peptide-to-lipid ratio. Note that the high sensitivity of ^{19}F NMR readily allowed us to address P/L as low as 1:800, using 0.1 mg of peptide and 12 h of instrument time. Each NMR sample was measured twice, first with its normal aligned parallel (0°) and then perpendicular (90°) to the static magnetic field. That way, the intragroup ^{19}F−^{19}F dipolar couplings could be analyzed in terms of peptide structure and mobility. Simple inspection of the ^{19}F NMR spectra already yields important information about the aggregation behavior of the epimeric MAP peptides in liquid crystalline DMPC. Basically, a narrow triplet indicates that the peptide assumes a well-structured and uniform conformation in the membrane. If a molecule undergoes fast long-axial rotation about the membrane normal, then its dipolar splitting at 0° has to be twice as large as for the 90° sample inclination. This behavior is observed for the *D*-epimeric peptide at all P/L ratios up to 1:25 (Figure 3, right panel). On the other hand, the *L*-epimer (left panel) shows motional averaging at P/L equal to 1:800 (and for a subpopulation at 1:400), while at higher peptide concentration a broad powder pattern is observed for all samples at 0° and 90° alignment. Notably, the occurrence of such powder line shape in the spectra acquired at 0° demonstrates that a peptide is not only immobilized but that it has also lost its unique structure or uniform alignment in the lipid bilayer. In other words, ^{19}F NMR of the CF_3−Phg labeled MAP analogue shows that the *L*-epimeric peptide starts to aggregate in DMPC at a concentration of P/L equal to about 1:400.

Having demonstrated by ^{19}F NMR and OCD that the *D*-epimeric peptides can be reconstituted in DMPC even at high concentration without aggregating, it is now possible to determine the helix alignment of MAP in the lipid bilayer. The ^{19}F dipolar splittings of all four MAP analogues that have been individually labeled with *D*-CF_3−Phg were collected in DMPC at 0° sample alignment (Supporting Information). These data are sufficient to calculate the helix tilt angle τ (inclination of the helix axis with respect to the membrane normal), the azimuthal rotation angle ρ (orientation of the polar face of the amphiphilic helix), and the order parameter S_{mol} (a measure of peptide wobble), as previously described in detail for other peptides.[22−25] For all labeled *D*-epimers the dipolar splittings are found to change as a function of concentration above a threshold P/L of 1:100. It is thus clear that MAP changes its alignment in the membrane, as it has been qualitatively observed above by OCD at a similar threshold (Figure 2B).

Figure 4 shows the helix alignment as derived from the *D*-epimeric MAP peptides at a P/L of 1:400 (Figure 4A,B) and 1:40 (Figure 4C,D). The tilt angle τ and the azimuthal rotation angle ρ are obtained as the best-fit solution upon screening the root-mean-square deviation (rmsd) between the experimental data and the dipolar splittings predicted for all angular combinations. The minima in the τ/ρ maps tell that at low concentration the

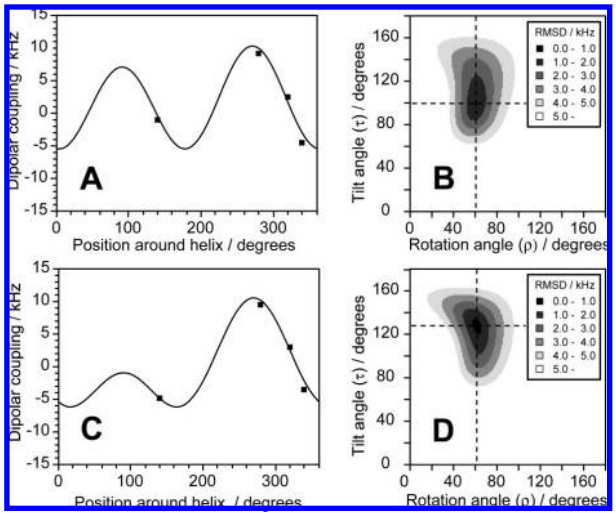

Figure 4. Dipolar wave analysis of the ^{19}F NMR triplet splittings of the four *D*-CF_3−Phg labeled MAP analogues at (A) P/L = 1:400 and (C) P/L = 1:40 together with the corresponding rmsd structure analysis (B, D) showing the helix tilt angle and azimuthal rotation of *D*-epimeric MAP in DMPC.

peptide helix is aligned almost flat in the membrane with a tilt angle τ of 100° relative to the bilayer normal. At higher peptide concentration the tilt increases to about 130°, with the C-terminus embedded more deeply in the hydrophobic bilayer core. At both concentrations, the polar face of the amphiphilic helix is aligned toward the aqueous phase ($\rho \approx 60°$), and the peptide wobble ($S_{mol} \approx 0.7$) is typical of a monomer or small oligomer. In fact, the concentration-dependent realignment of MAP is very similar to that described recently by solid state NMR for the two related antimicrobial peptides PGLa and MSI-103. They have a similar amphiphilic α-helical structure, which has been shown to realign from a monomeric surface-bound S-state to a tilted and presumably dimeric T-state. The only remarkable difference between these structurally related membrane-active peptides is the pronounced tendency of wild-type MAP (and its *L*-epimeric analogues) to aggregate extensively in the lipid bilayer. Using sterically restrictive *D*- and *L*-CF_3−Phg side chains, we were thus able not only to observe but also to prevent this effect, and to analyze the structure and realignment of the nonaggregating helical *D*-epimers. It will be interesting to find out whether and in which way the different aggregation behavior of the *D*- and *L*-epimeric MAP may influence their biological activities.

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Supporting Information Available: Experimental details and CD and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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