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Solid state NMR analysis of the dipolar couplings within and between distant CF_3-groups in a membrane-bound peptide

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Abstract

Dipolar couplings contain information on internuclear distances as well as orientational constraints. To characterize the structure of the antimicrobial peptide gramicidin S when bound to model membranes, two rigid 4-CF_3-phenylglycine labels were attached to the cyclic backbone such that they reflect the behavior of the entire peptide. By solid state ^{19}F NMR we measured the homonuclear dipolar couplings of the two trifluoromethyl-groups in oriented membrane samples. Using the CPMG experiment, both the strong couplings *within* each CF_3-group as well as the weak coupling *between* the two CF_3-groups could be detected. An *intra*-CF_3-group dipolar coupling of 86 Hz and a weak *inter*-group coupling of 20 Hz were obtained by lineshape simulation of the complex dipolar spectrum. It is thus possible to explore the large distance range provided by ^{19}F-labels and to resolve weak dipolar couplings even in the presence of strong *intra*-CF_3 couplings. We applied this approach to distinguish and assign two epimers of the labeled gramicidin S peptide on the basis of their distinct ^{19}F dipolar coupling patterns.

Keywords: Gramicidin S peptide; Carr–Purcell–Meiboom–Gill (CPMG) multipulse experiment; Oriented membrane sample; 4-Trifluoromethylphenylglycine; Homonuclear dipolar splitting

1. Introduction

Solid state NMR is a versatile tool to study biomembranes, as it can yield the full structure and dynamic behavior of membrane-active peptides in a quasi-native lipid environment [1]. ^{19}F-labeling of synthetic peptides offers the advantage of very high NMR sensitivity, strong dipolar couplings, and a lack of background signals [2–5]. Several ^{19}F-labeled peptides have been thoroughly characterized in terms of their conformation, membrane alignment and mobility [6–14]. Dipole–dipole couplings between two selective ^{19}F-labels provide a useful source of structural information, as their dependence on both internuclear distance and orientation in the magnetic field can be analyzed. Methods to measure dipolar couplings are well established and mostly based on magic angle spinning techniques (for reviews see, e.g., [15–20]). In particular, the strong dipolar couplings of ^{19}F make a large distance range accessible. In magic angle spinning experiments, distances of up to 12 Å have been reached, in fact employing CF_3-groups as fluorine labels [21]. These techniques, however, suffer from the loss of orientational information during sample spinning. As an alternative strategy, static multipulse experiments have been introduced, which can yield dipolar couplings in macroscopically oriented membrane samples [22–26]. With the aid of an improved Carr–Purcell–Meiboom–Gill (CPMG) sequence it was possible to isolate the homonuclear dipolar interaction from other interactions such as chemical shift, and this way to determine even weak dipolar couplings in aligned samples [27–31]. Using

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the CPMG experiment it has been possible to resolve a dipolar splitting of 100 Hz between two 4F-phenylglycine (4F–Phg)[1] labels on the antimicrobial peptide gramicidin S, embedded in oriented membranes [3,6,8].

Recently, the use of ^{19}F dipolar couplings in combination with oriented samples was extended from two spins to the symmetrical group of three spins within a trifluoromethyl moiety [32,33]. This strongly coupled triad offers itself as a formidable label to measure the orientation of a CF_3-group. Fast uniaxial rotation about the methyl axis makes the dipolar interaction solely dependent on the segmental orientation, and leads to favorable relaxation properties [12,34]. The dipolar splitting is not affected by the local molecular environment nor susceptibility effects, in contrast to the chemical shift anisotropy of a single ^{19}F-substituent which can be difficult to reference [35,36]. For this reason the CF_3-group often is a more advantageous label for measuring segmental orientations compared to a single fluorine-substituent [3–5]. Using the non-natural amino acid 4-trifluoromethyl-phenylglycine (CF_3–Phg) as a label, the structure of the antimicrobial peptide PGLa in membranes has been recently determined [10], and its concentration-dependent re-alignment was described [11,37]. In the simple 1-pulse experiments used in these investigations the sign of the dipolar splittings could be inferred from the corresponding CSA.

Here, the analysis of ^{19}F couplings in a CF_3-group is extended even further to include the coupling between two such CF_3-labels [12]. The strong couplings within each CF_3-group together with the weaker couplings between the two CF_3-groups give rise to a complicated dipolar spectrum. The dipolar pattern depends not only on the orientation of each CF_3-segment with respect to the magnetic field, but also on the distance between the two labeled groups as well as the orientation of their interconnecting vector. The feasibility of this approach has been recently demonstrated using aromatic model compounds bearing two CF_3-groups in a fixed geometry, which are embedded in oriented lipid membranes [12]. In the present study, we apply this strategy to a more complex biological system. To this aim, the cyclic decapeptide gramicidin S was labeled in two positions with CF_3–Phg, substituting the two symmetrically disposed valine residues (Fig. 1). The peptide was reconstituted in oriented liquid crystalline lipid bilayers to provide a functionally relevant environment comparable to a biological membrane. Using the CPMG experiment, it was possible to measure simultaneously both the strong *intra*-CF_3-group coupling as well as the much smaller *inter*-CF_3-group coupling. It is often argued that the observation of weak dipolar couplings can be hampered by the presence of strong couplings [38–41]. These difficulties, however, did not prevent the observation in this study of a small coupling of about 20 Hz in the presence of a more than fourfold stronger coupling.

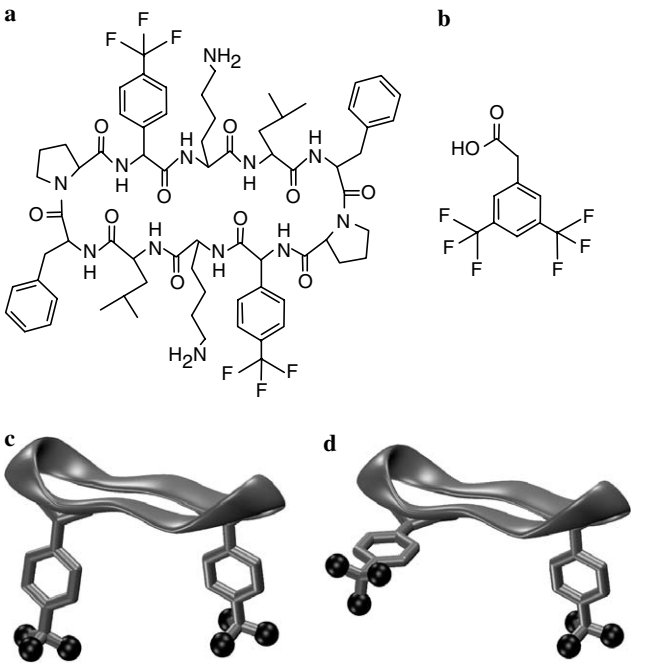

Fig. 1. Dipolar couplings were studied in two gramicidin S analogues (shown here is $(CF_3)_2$-GS/K) (a), and in the model compound bis-3,5-trifluoromethyl-phenylacetic acid ($(CF_3)_2$–PhA) (b). The peptide epimers possessing either two L-CF_3–Phg (c), or one L-CF_3–Phg and one D-CF_3–Phg (d), could be distinguished and assigned on the basis of their ^{19}F–^{19}F dipolar coupling patterns.

The analysis of ^{19}F dipolar couplings proved particularly useful in allowing us to distinguish and assign stereoisomers of gramicidin S. When synthesizing the labeled peptide, racemization of the CF_3–Phg residues is difficult to avoid. Peptide synthesis thus resulted in several epimeric peptides, differing in the L- or D-configuration of CF_3–Phg in each of the two labeled positions [14,42]. Although these diastereomers can be separated by chromatographic methods, the chemical derivatization and assignment of a peptide fraction to the specific epimer is tedious. However, on the basis of the measured ^{19}F dipolar couplings and the presence or absence of *inter*-residue couplings, an identification of the isomers became possible from first principles.

2. Materials and methods

2.1. Sample preparation

To label gramicidin S, both valines in the cyclic decapeptide sequence *cyclo*-[Pro-Val-Orn-Leu-D-Phe]$_2$ were substituted by 4-trifluoromethyl-phenylglycine (CF_3–Phg), giving "$(CF_3)_2$-GS." In a second analogue the two ornithines were additionally replaced by lysine ("$(CF_3)_2$-GS/K," see Fig. 1a) in order to examine the influence of the length

[1] *Abbreviations:* 4F–Phg, 4-fluoro-phenylglycine; CF_3–Phg, 4-trifluoromethyl-phenylglycine; CPMG, Carr–Purcell–Meiboom–Gill experiment; DMPC, dimyristoyl-phosphatidylcholine; $(CF_3)_2$-GS, gramicidin S substituted in both Val positions by CF_3–Phg; $(CF_3)_2$-GS/K, gramicidin S substituted in both Val positions by CF_3–Phg, and with both ornithines replaced by lysine; $(CF_3)_2$–PhA, bis-3,5-trifluoromethyl-phenylacetic acid.

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of the charged side-chain. The peptides were synthesized by solid phase Fmoc-protocols and purified as described previously [14,42]. A racemic mixture of D-/L-CF_3–Phg was employed, since phenylglycine would racemize anyway during synthesis. As a result, three stereoisomers were obtained, containing either two L-CF_3–Phg residues (LL-$(CF_3)_2$-GS, see Fig. 1c), two D-CF_3–Phg [DD-$(CF_3)_2$-GS), or one L-CF_3–Phg plus one D-CF_3–Phg (LD-$(CF_3)_2$-GS, see Fig. 1d). Due to the symmetry of the cyclic peptide, the mixed stereoisomer (LD = DL) was formed with twice the yield. The spatial structures of the LL- and LD-epimers are illustrated in Figs. 1c and d. The epimers were initially distinguished and assigned according to (i) their HPLC intensities and retention times, (ii) according to the products after peptide acid hydrolysis and derivatization with Marfey's reagent, and (iii) according to the number of lines in the ^{19}F NMR spectra in solution [14,42].

To prepare macroscopically oriented membrane samples for solid state ^{19}F NMR [6,10,11,14,31–33], 0.5–1 mg of peptide was co-dissolved with dimyristoyl-phosphatidylcholine (DMPC) in 300–450 μl chloroform/methanol (2:1) to give a peptide/lipid ratio of 1:20. The solution was spread onto 18 mm × 7.5 mm × 0.06 mm glass plates (Marienfeld, Lauda Königshofen), which were cleaned with chloroform and methanol before use. About 0.5–1 mg of material (dry weight) was deposited in 30 μl drops on each glass plate. After evaporation of the solvent and drying overnight under vacuum 10–15 plates were stacked, hydrated in a humid atmosphere (96% r.h. over K_2SO_4) at 48 °C for 24 h, and subsequently sealed in parafilm and plastic foil. The quality of alignment of the phospholipid bilayers was quantified by ^{31}P NMR, as seen in Fig. 2.

To establish a procedure for the data analysis of gramicidin S, the model compound bis-3,5-trifluoromethylphenylacetic acid (''$(CF_3)_2$–PhA,'' see Fig. 1b) was incorporated in DMPC bilayers at a molar ratio of ~1:7. An oriented membrane sample with $(CF_3)_2$–PhA was prepared following the same protocol as described above for the peptide.

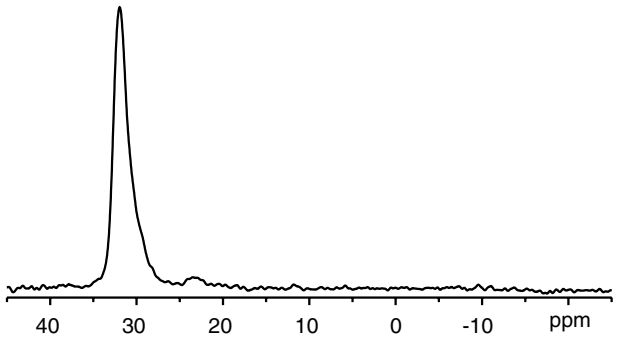

Fig. 2. The quality of the aligned membrane samples was checked by ^{31}P NMR. The example shown for $(CF_3)_2$-GS/K in DMPC confirms nearly 100% alignment (intensity at ~30 ppm), and almost no unoriented powder contribution (intensity at ~ −10 ppm).

2.2. NMR spectroscopy

To assess the quality of lipid orientation, ^{31}P NMR spectra were acquired on a Bruker Avance spectrometer operating at 202 MHz ^{31}P resonance frequency and 500 MHz ^{1}H frequency, using a Bruker triple resonance wideline probe equipped with a flat solenoid coil (Bruker, Karlsruhe). A Hahn echo sequence with a 90° pulse length of 6 μs and an echo delay time of 30 μs was employed for the ^{1}H-decoupled ^{31}P NMR spectra of oriented membranes aligned with their normal parallel to the magnetic field. ^{19}F NMR measurements were performed on the same spectrometer at 470 MHz, using a Doty 2.5 mm × 9 mm × 10 mm flat coil $^{19}F/^{1}H$ double resonance probe (Doty Scientific Inc., Columbia, SC). The samples were measured at 40 °C and were oriented with the normal of the membranes parallel to the magnetic field. Other sample orientations were not used as the studied molecules are known to perform fast axially symmetric motions around the membrane normal under the employed experimental conditions [6,43]. Simple 1-pulse experiments with a 90° pulse width of 2.4 μs and 25 kHz TPPM ^{1}H-decoupling were used to acquire the regular ^{19}F NMR spectra, referenced relative to $CFCl_3$. Dipolar spectra were acquired using a modified CMPG sequence with $90°_{\phi\ -\ 90°}\ 180°_{\phi}\ 90°_{\phi\ -\ 90°}$ composite pulses replacing the $180°_{\phi}$ pulses of the basic CPMG experiment [32,44]. The phases of the refocusing pulse were alternated in an xy8 pattern [30,31,45] provided that the required spectral range allowed a reduced sweep width. The CPMG pulse trains consisted of 1280 pulses of 9.6 μs pulse length (corresponding to a 90° pulse length of 2.4 μs) with interpulse delays of 40 μs. Stroboscopic data sampling between the pulses was applied, and 2–4 data points were acquired in the interpulse delays (or in each 4th delay when the xy8 phase cycle was applied) and averaged. No ^{1}H-decoupling was employed during the CPMG pulse train. The finite pulses lead to a scaling of the CPMG spectra by a factor which depends on the duty cycle Q (defined as the fraction of the time of radiofrequency-irradiation during the pulse train) [29,32]. A scaling factor of $(1 - 9/8Q)$ was calculated for the limit of small duty cycles from 0th order Hamiltonian theory for $90°_{\phi\ -\ 90°}\ 180°_{\phi}\ 90°_{\phi\ -\ 90°}$ composite pulses and xy8 phase alternation (see Appendix A), which is in good agreement with the observed splittings of $(CF_3)_2$–PhA acquired in a series of varying duty cycles (data not shown). The frequency scales of all spectra displayed here were multiplied by $1/(1 - 9/8Q) = 1.278$, resulting from the duty cycle $Q = 0.1935$, to correct for this scaling behavior.

2.3. Data analysis

If there are both kinds of coupling present, within as well as between the two CF_3-groups, it is generally not possible to determine the dipolar couplings directly from the multiplet spectrum. In the case of LL-$(CF_3)_2$-GS and LL-$(CF_3)_2$-GS/K, the experimental CPMG spectra were there-

fore simulated and fitted using the GAMMA program library [46]. From earlier ^{19}F NMR studies it is known that gramicidin S adopts a flat alignment on the membrane surface, with its twofold symmetry axis parallel to the bilayer normal [6]. It is therefore justified to assume that the alignment of the two CF_3-groups with respect to the membrane normal is symmetrical, hence identical *intra*-CF_3 couplings can be expected for the two groups. For the data analysis, we have thus characterized the entire 6-spin system by a single *intra*-CF_3-group coupling D, plus one *inter*-CF_3-group coupling d. They are defined by the homonuclear dipolar interaction Hamiltonian

$$H = -D\sum_{k=1}^{2}\sum_{l=k+1}^{3}(3I_{z,k}I_{z,l} - \mathbf{I}_k\mathbf{I}_l) - D\sum_{k=4}^{5}\sum_{l=k+1}^{6}(3I_{z,k}I_{z,l} - \mathbf{I}_k\mathbf{I}_l) - d\sum_{k=1}^{3}\sum_{l=4}^{6}(3I_{z,k}I_{z,l} - \mathbf{I}_k\mathbf{I}_l), \quad (1)$$

where the first and second term describe the *intra*-CF_3 couplings of spins within the two triads (labeled 1–3 and 4–6, respectively), and the third term adds contributions from *inter*-CF_3-group couplings. Both couplings D and d correspond to the zz-components of the respective time-averaged dipolar coupling tensors. These tensors depend not only on the distances and relative orientations of the spin pairs involved, but also on the dynamics and geometry of the averaging motion. Both coupling tensors can be assumed axially symmetric, which for the *inter*-CF_3 coupling is not generally the case (since here the ^{19}F–^{19}F coupling is averaged by motion lacking axially symmetry), but which is a valid approximation for distant CF_3-groups.

The ideal multiplet lineshape resulting from the interaction of the spin system is broadened in the experimental spectra in a frequency-dependent way, with increased broadening in the outer regions of the CPMG spectra. To fit by calculated spectra optimally, such differential linebroadening was approximated in the simulated spectra in two ways. An overall linebroadening was applied by multiplying the calculated FID with an exponential prior to Fourier transformation. The frequency-dependent contribution to the broadening was attributed mainly to imperfect molecular alignment. The resulting effect on the lineshape was approximated by averaging 10,000 spectra, scaled by $(3\cos^2\beta - 1)/2$ with an alignment angle β randomly distributed around 0 with a distribution function $\sim\sin(\beta)\exp(-\beta^2/2\sigma)$. The mosaic spread σ was adapted to the experimental spectra and varied between 7° and 30°.

To identify the best fit between the simulated and experimental CPMG spectra, a grid search was performed. To this aim, spectra were calculated and compared with the experimental data for all combinations of *intra*-CF_3-group and *inter*-CF_3 group couplings in the range of $-180\text{ Hz} < D < 180\text{ Hz}$ and $-30\text{ Hz} < d < 30\text{ Hz}$ ($-240\text{ Hz} < D < 240\text{ Hz}$ and $-100\text{ Hz} < d < 100\text{ Hz}$ in the case of the model compound), using step sizes between 1 Hz and 4 Hz. The quality of the fit was quantified by calculating the point-by-point rmsd of simulated and experimental spectra for each pair of *intra*- and *inter*-CF_3-group couplings. Fit minima were accepted if the rmsd was not more than 20% larger than the lowest rmsd value obtained in the entire grid search. The errors of the obtained dipolar couplings were estimated from the region in the rmsd plot with rmsd values exceeding the corresponding local minimum by less than 10%. A more rigorous derivation of error limits on statistical grounds would have required that noise is the dominant contribution to the deviation between best fit spectra and experimental spectra, which was not the case.

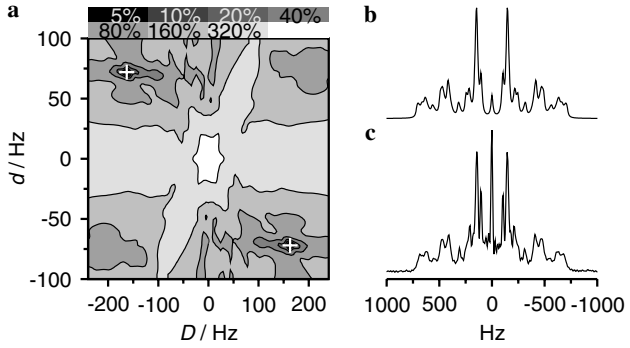

Fig. 3. The fitting procedure was validated using the model compound $(CF_3)_2$–PhA carrying two CF_3-groups (see Fig. 1b). Simulated and experimental spectra were compared on the basis of root mean square deviations (rmsd) by systematically varying the *intra*- and *inter*-CF_3 couplings D and d (a). The best fit (b) found this way for $D = +(-)163$ Hz and $d = -(+)72$ Hz reproduces the experimental spectrum (c) in all detail. (The percentages for the contour levels refer to the deviation from the global rmsd minimum, which are marked by +.)

To validate this fitting procedure, we first analyzed the 6-spin couplings of the model compound $(CF_3)_2$–PhA (see Fig. 1b) embedded in oriented DMPC membranes, since its well-resolved dipolar pattern allows an assessment of the fit quality. The best fit derived this way is shown in Fig. 3b. It clearly reproduces the experimental CPMG spectrum (Fig. 3c) very well, and the resulting peak frequencies are accurate to within ~10 Hz on average. From the rmsd analysis (Fig. 3a) an *intra*-CF_3-group coupling $D = \pm(163 \pm 6\text{ Hz})$ and an *inter*-CF_3-group coupling of $d = \pm(72 \pm 3\text{ Hz})$ were determined as defined by Eq. (1), in good agreement with our recent study [12]. Note that the centermost peak in the spectrum is an experimental artifact often found in CPMG spectra, due to incomplete refocusing or caused by a DC offset, and it is thus not reproduced to the same extent in the simulated lineshape.

3. Results

3.1. Dipolar spectra

To be able to analyze the wealth of structural information inherent in the NMR data, it is generally necessary to separate the different contributions of the spin interactions, such as chemical shift or dipolar couplings, to yield

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an interpretable NMR spectrum. The most prominent experimental schemes use magic angle spinning combined with recoupling pulses to selectively average out the undesired contributions [15–20]. As these approaches are not compatible with the use of oriented membrane samples, we instead employed a selective averaging technique based only on a static multipulse sequence. With the aid of a modified Carr–Purcell–Meiboom–Gill experiment [5,31–33] the chemical shift and heteronuclear dipolar couplings were removed, leaving only the homonuclear ^{19}F–^{19}F dipolar couplings to contribute to the spectrum.

The effect of the CPMG experiment becomes evident in the spectra of the two CF_3–Phg-labeled gramicidin S analogues LL-$(CF_3)_2$-GS/K and LL-$(CF_3)_2$-GS (see Fig. 1). Fig. 4 shows that the simple 1-pulse ^1H-decoupled ^{19}F NMR spectra without multipulse averaging (Figs. 4a and c) are featureless, and the ^{19}F–^{19}F dipolar couplings are not resolved. In the CPMG spectra (Figs. 4b and d), on the other hand, a pattern originating from the dipolar coupling is clearly visible. At first glance, the spectrum resembles the triplet expected for an isolated CF_3-group. Indeed, a triplet splitting of $\Delta_{obs} = 220$ Hz and $\Delta_{obs} = 290$ Hz is observed for LL-$(CF_3)_2$-GS/K and LL-$(CF_3)_2$-GS, respectively. Inspecting the lineshape more closely, however, deviations from a simple 1:2:1 triplet are noticed. In fact, the resolution is good enough to reveal an additional splitting of δ_{obs}=50 Hz across the central peak. Though the full details of the complex spectral pattern expected for the 6-spin system are not resolved, this splitting is a clear indication of a long-range coupling between the two CF_3-groups that are apposed in the same molecule. We also note at this point that the CPMG spectra of the two gramicidin S analogues $(CF_3)_2$-GS/K and $(CF_3)_2$-GS exhibit somewhat different widths in their outer spectral components, suggesting slight differences in the peptide backbone conformation due to the replacement of ornithine by lysine.

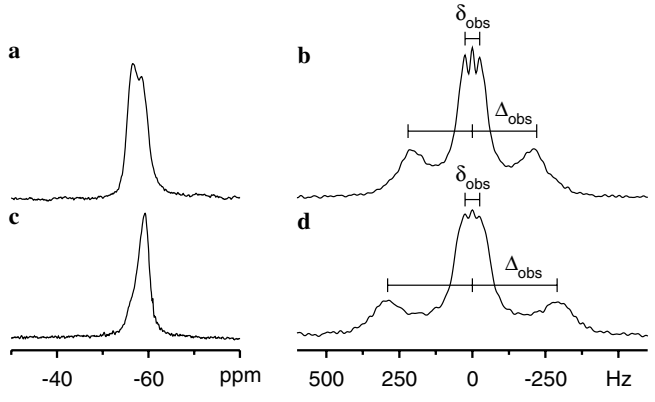

Fig. 4. ^{19}F NMR spectra of LL-$(CF_3)_2$-GS/K (a and b) and LL-$(CF_3)_2$-GS (c and d). In the spectra acquired after a single excitation pulse with ^1H-decoupling, the dipolar couplings are not resolved and obscured by the chemical shift (a and c). Using the CPMG multipulse sequence without ^1H-decoupling, details of the 6-spin dipolar spectrum become visible (b and d). Overall, the CPMG spectra possess a triplet-like pattern with splittings Δ_{obs}. In the center a further splitting δ_{obs} is observed, indicating the presence of a weak dipolar coupling between the two CF_3-groups.

3.2. Lineshape simulations

The CPMG spectra in Figs. 4b and d differ from the simple dipolar lineshape of a single CF_3-group, and do not correspond to a mere superposition of the spectra of two isolated CF_3-groups. In this case of two *coupled* CF_3-groups, the dipolar coupling values cannot generally be determined directly from the splittings seen in the spectra. Therefore, to analyze the CPMG data, dipolar spectra of the underlying 6-spin system were simulated for the LL-gramicidin S analogues and fitted to the experimental lineshapes. In contrast to our recent study of small CF_3-labeled model compounds [12], the 6-spin dipolar pattern of gramicidin S is not fully resolved in the larger and less mobile peptides studied here. For this reason, the simulated and experimental lineshapes themselves were compared in a grid search, rather than comparing the respective peak frequencies.

The lineshape fitting algorithm has been demonstrated to work reliably on the model compound (see Section 2) and is now applied to the CPMG spectra of LL-$(CF_3)_2$-GS/K and LL-$(CF_3)_2$-GS. The resulting rmsd plot for LL-$(CF_3)_2$-GS/K in Fig. 5a shows four minima with similar rmsd values for pairs of *intra*- and *inter*-CF_3-group couplings D and d. The solutions are $(D_1,\ d_1) = (86$ Hz, 20 Hz), $(D_2,\ d_2) = (-85$ Hz, 19 Hz), $(D_3,\ d_3) = (85$ Hz, -19 Hz), $(D_4,\ d_4) = (-86$ Hz, -20 Hz), with errors of 8 Hz for D and 2 Hz for d. The two minima at $(D_1,\ d_1)$ and $(D_4,\ d_4)$ (marked with × in Fig. 5a), as well as those at $(D_2,\ d_2)$ and $(D_3,\ d_3)$ (marked with + in Fig. 5a), are mutually equivalent, since only the relative sign of the two dipolar couplings can be distinguished, and the dipolar spectrum is invariant to a simultaneous sign inversion of all contributing dipolar terms. The best fit lineshapes corresponding to the two possibilities are depicted in Figs. 5b and c. Both reproduce the experimental spectrum (Fig. 5d) very well, including its central region. Linebroadening and the mosaic spread of the molecular alignment partially obscure the details of the underlying spectrum of the 6-spin system. These effects were taken into account in the simulations to reproduce the experimental data correctly (see Section 2.3). Spectra calculated with reduced linebroadening and without mosaic spread (gray stick spectra in Fig. 5) show the underlying dipolar pattern.

From our previous knowledge of the alignment of gramicidin S in membranes, it is possible to select the most likely solution from the four possibilities above, using geometrical arguments [6]. We know that the interconnecting CF_3–CF_3 vector of the symmetrical molecule is aligned on average at a 90° angle with respect to the membrane normal because the peptide backbone binds flat to the lipid bilayer. Hence, the sign of the *inter*-CF_3-group coupling d must be negative, due to its orientation dependence according to $3[\cos^2(90°) - 1]/2 = -1/2$. The sign of D depends on

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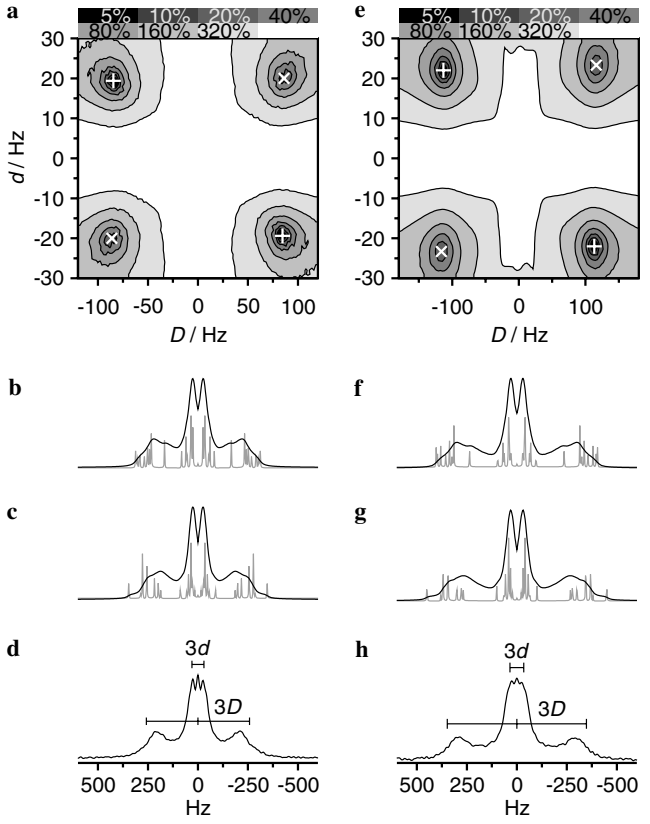

Fig. 5. With the aid of a grid search (as validated in Fig. 3), four pronounced rmsd minima were found for $(CF_3)_2$-GS/K (a). Because only the relative sign of the respective couplings can be distinguished, the rmsd minima occur as pairs with identical spectra. The best fit spectra for $D = +(-)85$ Hz, $d = -(+)19$ Hz ((b), minima indicated by + in (a)) and for $D = +(-)86$ Hz, $d = +(-)20$ Hz ((c), minima indicated by × in (a)) are in good agreement with the experimental spectrum (d). In the rmsd analysis for $(CF_3)_2$-GS (e), four minima were obtained with $D = +(-)113$ Hz, $d = -(+)22$ Hz ((f), minima indicated by + in (e)) and at $D = +(-)116$ Hz, $d = +(-)23$ Hz ((g), minima indicated by × in (e)). As for $(CF_3)_2$-GS/K, the best fit spectra are in good agreement with the experimental spectrum (h). The underlying dipolar pattern of the 6-spin spectra becomes visible if linebroadening is reduced and mosaic spread omitted in the simulation (gray stick spectra). The broadened spectra exhibit splittings which are close to the limiting value of $3D$ expected for an isolated CF_3-group coupled only by D, and to the value of $3d$ that would be expected for a simple spin pair coupled by d (indicated in (d) and (h)).

the effective orientation of the *intra*-CF_3 dipolar interactions and includes two factors. First, fast rotation of the CF_3-group introduces a negative sign, since the pair-wise dipolar interactions are orthogonal to the averaging CF_3-axis. Second, the alignment of the CF_3-group, which is collinear with the Cα–Cβ bond, is expected to be more or less parallel to the membrane normal given the conformation of the cyclic peptide (see Fig. 1c). Since this expected angle is smaller than the magic angle, the negative sign of D should be maintained. We thus conclude that $d < 0$ and $D < 0$, leaving the solution of $D_4 = -86$ Hz and $d_4 = -20$ Hz as the most likely minimum.

A similar analysis was performed for LL-$(CF_3)_2$-GS, as illustrated in Figs. 5e–h. Again, four minima were obtained in the rmsd analysis of the CPMG spectrum for dipolar couplings of $(D_1, d_1) = (116$ Hz, 23 Hz), $(D_2, d_2) = (-113$ Hz, 22 Hz), $(D_3, d_3) = (113$ Hz, -22 Hz), $(D_4, d_4) = (-116$ Hz, -23 Hz).

3.3. Distinguishing different epimers of gramicidin S

In the course of peptide synthesis, three different epimers were obtained for each gramicidin S analogue due to the racemic CF_3–Phg. They contain either two L-forms (LL), two D-forms (DD) or one L- and D-form each (LD) of CF_3–Phg (see Figs. 1c and d). The peptide epimers could be easily separated by HPLC, but a firm assignment of the different fractions has been awkward so far [14,42]. With the aid of ^{19}F–^{19}F couplings, we are now able to confirm our assignment of the LL- and LD-epimers on the basis of first principles. The DD-fraction was not included in this study, as it was readily identified on grounds of low synthesis yield (since structural distortions probably prohibited successful cyclization of the linear precursors [42]).

Inspection of the gramicidin S model structures in Fig. 1 shows that the two CF_3-groups are symmetrically and rather closely apposed in the LL-epimer (Fig. 1c), whereas they are skewed and rather distant in the LD-form of the peptide (Fig. 1d). Indeed we observed distinctly different types of ^{19}F NMR spectra for the LL- and LD-epimers of $(CF_3)_2$-GS/K and $(CF_3)_2$-GS in model membranes, of which the LL-forms has been discussed above. Fig. 6 illustrates that the LD-epimers give rise to large spectral widths, covering about 30 ppm in the single-pulse ^{19}F-spectra (Figs. 6a and c) and 6 kHz in the CPMG spectra (Figs. 6b and d). In contrast, the spectra of the corresponding LL-peptides in Fig. 4 only cover a range of approximately 5 ppm in

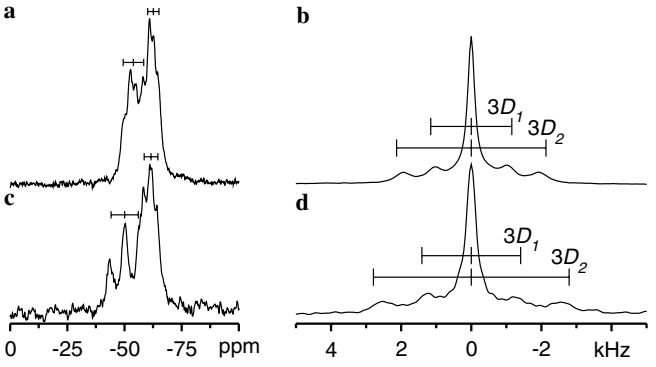

Fig. 6. ^{19}F-spectra of LD-$(CF_3)_2$-GS/K (a and b) and LD-$(CF_3)_2$-GS (c and d). The spectra acquired after a single excitation pulse with ^1H-decoupling show a convolution of dipolar couplings and chemical shift (a and c). Using the CPMG sequence, the dipolar spectrum composed of a superposition of two triplets with different couplings D_1 and D_2 is revealed (b and d). The respective splittings are extracted by fitting the CPMG spectra, giving $3D_1 = 1.16$ kHz and $3D_2 = 2.13$ kHz for $(CF_3)_2$-GS/K (b), and $3D_1 = 1.41$ kHz and $3D_2 = 2.79$ kHz for $(CF_3)_2$-GS (d). These values are indicated by scale bars in the CPMG and single-pulse spectra. The differing *intra*-CF_3-group couplings and the absence of an *inter*-CF_3-group coupling allows an assignment of these molecules to the asymmetric LD-epimeric form of gramicidin S.

the single-pulse spectra, and 1 kHz in the CPMG spectra. Furthermore, the small splitting of the central component in the CPMG spectra of the LL-analogues (Figs. 4b and d) is absent in the spectra of the LD-analogues (Figs. 6b and d). Instead, a single central peak and two large splittings are observed in the CPMG spectra of LD-$(CF_3)_2$-GS/K and LD-$(CF_3)_2$-GS. This pattern represents a superposition of two triplets originating from two isolated CF_3-groups, which are too far apart to engage in any detectable coupling d. The corresponding splittings extracted from fitting the CPMG spectra are $3D_1 = 1.16$ kHz and $3D_2 = 2.13$ kHz for $(CF_3)_2$-GS/K (Fig. 6b), and $3D_1 = 1.41$ kHz and $3D_2 = 2.79$ kHz for $(CF_3)_2$-GS (Fig. 6d). The different coupling values D within the same spectrum indicate a different alignment of the two CF_3-groups in the membrane, as expected from the asymmetric molecular structure of the LD-epimer (Fig. 1d).

We also note that the single-pulse spectra show features of a set of two triplets which are convoluted with the chemical shift (Figs. 6a and c). The triplet splittings revealed in the CPMG experiment (indicated by scale bars in Fig. 6) can be clearly identified in the one-pulse spectra of both peptides. Furthermore, in the case of $(CF_3)_2$-GS both triplets are resolved, and the splittings read from the 1-pulse spectrum (1.4 kHz and 3.2 kHz) are close to the values derived from the CPMG spectrum. From these parameters a clear distinction of the LL- and LD-epimers was possible and confirms our previous assignment [14,42].

4. Discussion

The aim of our study was to validate a new strategy to enhance the number of structural parameters obtained from ^{19}F NMR dipolar coupling measurements. In the antimicrobial peptide gramicidin S two CF_3-groups were used as labels, and their ^{19}F–^{19}F dipolar couplings were measured in oriented membrane samples with the aid of a CPMG experiment. Besides the strong *intra*-CF_3-group coupling D, also the weak coupling d between the two CF_3-groups was observed. This way not only orientational information from the *intra*-CF_3-group D couplings can become accessible, but d can reveal additional structural information on the distance between the labels and the alignment of their *inter*-connecting vector. In principle the *inter*-CF_3 coupling d could also originate from fluorines in different gramicidin S molecules. However, an *inter*-molecular coupling seems unlikely inlight of the clearly defined features observed for the dipolar coupling, which indicate a narrow distribution of dipolar couplings. Unless specific peptide oligomerization plays a role (and which was not observed under the used experimental conditions [6,43]), any potential proximity of peptide molecules in the laterally unordered membrane environment would more likely result in a distribution of *inter*-molecular CF_3-distances, which would lead to unspecific broadening of the spectra. The detection of weak dipolar interactions in the presence of strong dipolar couplings is often thought to be hampered due to truncation by the strong contribution [38–41]. Nonetheless, two couplings differing by a factor of more than 4 were simultaneously measured here. A splitting of $\delta_{obs} = 50$ Hz was resolved, even though the experimental lineshapes are distorted by a central spike which is a typical artifact in CPMG spectra. One reason why the detection of weak couplings was facilitated here might be the advantageous relaxation properties of the CF_3-groups due to fast methyl rotation, which reduces T_2 relaxation and increases resolution. The high mobility of a molecule in a liquid crystalline membrane also seems to contribute to this improvement, as evident from the better resolution obtained with the small model compound compared to the peptide.

The broadened CPMG spectra of the two peptides (Figs. 5d and h) exhibit the typical features of a dipolar pattern expected for the limit of $D \gg d$. The dominant *intra*-CF_3-group coupling D produces a triplet, whose lines are further split by the weak *inter*-CF_3-group coupling d. In this case, we see that the observed splittings Δ_{obs} and δ_{obs} (Figs. 4b and d) are close to the values of $3D$ and $3d$, respectively. A splitting of $3D$ would correspond to the limit of an isolated CF_3-group, and a splitting of $3d$ would correspond to the situation of a simple pair of two spins. (We had previously defined these splittings as $\Delta_{CF3} = 3D$ and $\Delta_{FF} = 3d$, see e.g., [5].) For example, the splitting $\Delta_{obs} = 220$ Hz directly visible in the CPMG spectra of $(CF_3)_2$-GS/K (Fig. 4b) is similar to the value of $3D = 258$ Hz, if approximating the spectrum by a triplet spectrum with the coupling of $D = 86$ Hz as determined by fitting. Likewise, the apparent central splitting of $\delta_{obs} = 50$ Hz is close to the value of $3d = 60$ Hz that would emerge from a spin pair coupled by a dipolar coupling d. Nonetheless, we note that the simplistic appearance discussed here stems from the broadening of the more complex underlying dipolar patterns consisting of a multitude of lines. To analyze these spectra in detail, it was essential to use computer simulations for extracting the accurate dipolar coupling values. Since not all lines are resolved in the present example, it was necessary to fit the lineshape amplitudes as a function of frequency rather than individual line positions. Furthermore, the effects of linebroadening and mosaic spread had to be taken into account, as they lead to a differential broadening of the lines with sharp features only in the center. The characteristic spectral frequencies read directly from the broadened spectra thus appear to be lower than the corresponding exact values determined by fitting.

To assign the LL- and LD-epimers of gramicidin S, clues can be derived from both, the *intra*-CF_3-group couplings D reflecting the side-chain orientations, and from the weak *inter*-CF_3-group coupling d reflecting mainly the CF_3–CF_3 distance. Given that the cyclic peptide backbone is stabilized by intramolecular hydrogen bonds, the L-enantiomer of a CF_3–Phg side-chain will be aligned with its C_α–C_β axis approximately perpendicular to the β-sheet plane (Fig. 1c). The corresponding D-amino acid, on the other hand, will be

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pointing out sideways (Fig. 1d). The amphiphilic gramicidin S molecule was previously shown to lie flat on the surface of the membrane under the conditions studied here. Thus, for the LL-analogues both CF_3–Phg side-chains must be aligned the same way in oriented membrane samples, and should give identical couplings D, which they indeed do. On the other hand, the two CF_3-groups in the skewed LD-analogues are expected to point asymmetrically into different directions, and they should thus give rise to two distinct couplings D_1 and D_2, which is indeed observed. Another consequence of the different side-chain orientations is a shorter CF_3–CF_3 distance and therefore stronger *inter*-CF_3-group coupling in the LL-analogue compared with the LD-case. These differences are indeed manifest in the CPMG spectra. While the central components of the spectra of the putative LL-analogues are characterized by a weak CF_3–CF_3 coupling d (Fig. 4), such coupling is far too weak to be noticed in the spectra of the LD-peptides (Fig. 6). At the same time, the CPMG spectra which do not exhibit any *inter*-CF_3-group coupling d, are instead superpositions of two triplets with different *intra*-CF_3-group couplings D_1 and D_2. Thus, by combining the information from both, *inter*- and *intra*-CF_3-group couplings, a direct assignment of the gramicidin S epimers was possible. The possibility of determining precise structural information of the chemically different $(CF_3)_2$-GS and $(CF_3)_2$-GS/K analogues is not attempted here, since the respective ^{19}F NMR data (Figs. 4b and d) suggest subtle differences in their backbone conformation. The cationic side-chain Orn in $(CF_3)_2$-GS differs only by one methylene unit from Lys in $(CF_3)_2$-GS/K, however, it seems that the peptide responds by slight changes in its backbone torsion angles. Hence a complete geometric analysis, which also has to take motional averaging effects into account, seems too ambitious for the time being. Nonetheless, the qualitative assessment has already brought valuable insight into the system, as demonstrated by the assignment of the LL-and LD-epimers.

Despite the benefits offered by the use of CF_3-groups as NMR labels, such as signal contributions of three spins, strong intrinsic couplings independent of the local environment, and advantageous relaxation properties, the use of more complex spin systems also bears disadvantages. In particular, the large number of spectral lines arising from the dipolar coupling network reduces the intensity of the individual peaks. Also, the resolution requirements get substantially more challenging as the number of lines increases more than linearly with the number of spins. Furthermore, the interpretation of the dipolar couplings becomes more involved with a larger number of spins, as coupling values can no longer be extracted from the spectra without simulation. The interpretation of the couplings may also bear some caveats in cases where the *inter*-CF_3-group coupling d approaches the same magnitude as the *intra*-CF_3-group couplings D. If this situation happens to be encountered in certain molecular alignments, then the averaging of the individual substituents by methyl rotation would have to be considered explicitly.

Nevertheless, in the limit of $d \ll D$ these disadvantages are only of minor concern. As seen in the experimental CPMG spectra of gramicidin S, the *inter*-CF_3-group coupling is manifested primarily as a splitting in the centre of the roughly preserved overall triplet lineshape. Thus the coupling did not cause the spectrum to break up into a smeared-out distribution of peaks, but it has lead to additional, characteristic features which could still be quantified. Also, an interpretation of the *inter*-CF_3-coupling in terms of a distance between the centers of the two CF_3-groups is possible to a good approximation if the CF_3–CF_3 distance is large compared to the ^{19}F–^{19}F distance within one CF_3-group. In labeling schemes of biological macromolecules we are most likely to encounter a large distance between the labels compared to the size of the CF_3-label itself. Hence, the opportunity of measuring additional structural constraints is usually anticipated to outweigh the potential disadvantages of CF_3-groups.

5. Conclusions

An advanced use of CF_3-groups as highly sensitive ^{19}F NMR-labels was demonstrated here. The dipolar couplings within a single CF_3-group are well suited to measure the segmental orientation in macroscopically aligned samples, such as oriented membranes [10,11,32–34,37]. In addition to this local orientational information, CF_3-groups were also shown here to provide a valuable source for distance information as well. We were able to measure the weak dipolar coupling between two distant CF_3-groups in the antimicrobial peptide gramicidin S even in the presence of nearly fivefold stronger couplings within the CF_3-groups. This approach opens up new opportunities to combine structural parameters from multiple CF_3-labels, as utilized in this case for distinguishing two epimeric peptides.

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Appendix A

Due to finite pulse lengths, the dipolar interaction observed with the CPMG sequence is scaled. The scaling factor depends on the duty cycle Q of the pulse train, but also on the pulses used. With 180° pulses as in the original CPMG sequence, the scaling factor is $(1 - Q)$ [29]. However, the composite pulses and xy8 phase alternation results in a different scaling factor. Using 0th-order average Hamiltonian theory, the scaling factor can be approximated as follows.

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Considering a pair of spins, the homonuclear dipole–dipole interaction is

$$H_{\mathrm{DD}} = -D(3I_{z,1}I_{z,2} - \mathbf{I}_1\mathbf{I}_2)$$

with the dipolar coupling D. With the Hamiltonian $H_1(t)$ describing the radiofrequency irradiation in the Zeeman rotating frame, the dipolar interaction in the interaction frame of $H_1(t)$ becomes

$$\tilde{H}_{\mathrm{DD}}(t) = U^{-1}(t)H_{\mathrm{DD}}U(t) \quad \text{with}$$

$$U(t) = \hat{T}\exp\left(-\mathrm{i}\int_0^t H_1(t)\mathrm{d}t\right).$$

The averages of the dipolar interaction $\tilde{H}_{\mathrm{DD}}(t)$ during the different elements of the pulse train can be calculated by integration and are in the case of $90°_{-y}\ 180°_{x}\ 90°_{-y}$ pulses: H_{DD} between the pulses, $\frac{1}{2}D(3I_{y,1}I_{y,2} - \mathbf{I}_1\mathbf{I}_2)$ for both flanking $90°_{-y}$ pulses of the composite pulse and $-D(3I_{x,1}I_{x,2} - \mathbf{I}_1\mathbf{I}_2)$ for the sandwiched $180°_{x}$ pulse. For $90°_{x}\ 180°_{y}\ 90°_{x}$ pulses the respective averages are H_{DD}, $\frac{1}{2}D(3I_{x,1}I_{x,2} - \mathbf{I}_1\mathbf{I}_2)$, $-D(3I_{y,1}I_{y,2} - \mathbf{I}_1\mathbf{I}_2)$. Simple averaging over the pulse train, taking into account the xy8 phase alternation, yields the 0th order average Hamiltonian

$$\bar{H}_{\mathrm{DD}} = \left(1 - \tfrac{9}{8}Q\right)H_{\mathrm{DD}}.$$

Hence, the CPMG pulse train with $90°_{\phi\ -\ 90°}\ 180°_{\phi}\ 90°_{\phi\ -\ 90°}$ composite pulses and xy8 phase alternation yields an average dipolar interaction scaled by $(1 - 9/8Q)$.

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