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Evaluating the amino acid CF_3-bicyclopentylglycine as a new label for solid-state ^{19}F-NMR structure analysis of membrane-bound peptides‡

SERGII AFONIN,[a] PAVEL K. MIKHAILIUK,[b] IGOR V. KOMAROV[b] and ANNE S. ULRICH[a,c]*

[a] Institute of Biological Interfaces, Forschungszentrum Karlsruhe, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[b] Department of Chemistry, Kyiv National Taras Shevchenko University, Vul. Volodymyrska 64, 01033 Kyiv, Ukraine
[c] Institute of Organic Chemistry, Universität Karlsruhe (TH), Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany

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Abstract: The conformation, alignment and dynamic behavior of membrane-bound peptides is readily accessible by solid-state ^{19}F-NMR spectroscopy, but it has been difficult to incorporate suitable ^{19}F-labelled amino acids into synthetic peptides. To avoid the drawbacks of previously used labels, we have rationally designed and synthesized a novel amino acid that suits all theoretical and practical requirements for peptide synthesis and subsequent ^{19}F-NMR structure analysis [Mikhailiuk *et. al*, Angew. Chem. 2006, 118, 5787–5789]. The enantiomerically pure L-form of 3-(trifluoromethyl)bicyclopent-[1.1.1]-1-ylglycine (CF_3-Bpg) carries a CF_3 group that is rigidly attached to the peptide backbone and does not racemize during peptide synthesis. It could be demonstrated for several different peptides that their biological activity is usually not affected by a single label, nor the conformation, as monitored by circular dichroism. Here, we carry out a more detailed structure analysis to evaluate the potential and reliability of CF_3-Bpg for solid-state NMR, using the well-known α-helical antimicrobial peptide PGLa as a test case. We have collected several orientational constraints from the anisotropic ^{19}F–^{19}F dipolar couplings of CF_3-Bpg in various positions of PGLa embedded in lipid bilayers. These resulting structural parameters are then compared with those previously determined from 4-CF_3-phenylglycine and 3,3,3-d_3-alanine labels on the same peptide. The analysis confirms that CF_3-Bpg does not perturb the α-helical conformation of PGLa. Likewise, the helix alignment is shown to follow the established concentration-dependent pattern in realigning from a surface-bound S-state to an obliquely tilted T-state. Hence, the advantages of CF_3-Bpg over all previously used ^{19}F-labeled side chains are evident, as they combine ease of chemical incorporation and peptide purification with high NMR sensitivity and absent background signals, allowing a straightforward analysis of the dipolar splittings with no need for chemical shift referencing without any ambiguity in the sign of the couplings. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-state ^{19}F-NMR; side-chain CF_3-labels; antimicrobial peptide PGLa; DMPC lipid membranes; homonuclear fluorine dipolar couplings; helix alignment

INTRODUCTION

Solid-state NMR offers a versatile and comprehensive strategy for determining the structure of small membrane-active peptides, such as antimicrobial, cell-penetrating, fusogenic sequences, peptidic hormones and toxins, as well as small transmembrane proteins [1–4]. Most of these systems are intrinsically dynamic or conformationally flexible, and they often tend to form specific assemblies above certain threshold concentrations. In such cases, both the low- and high-concentration states may be of functional relevance and need to be characterized in their native lipid environment. Such situations cannot be meaningfully studied by crystallography or electron microscopy, or by liquid-state NMR in micelles. Other approaches like EPR or fluorescence spectroscopy are appropriate for membrane systems, but they involve comparatively large labels and do not reach atomic resolution.

Solid-state NMR structure analysis is conceptually simple, as specific isotope labels are placed into representative sites of the synthetic sequence to provide orientational and/or distance constraints. Typically, several individually labeled samples are required to collect a number of constraints, from which the peptide conformation can be deduced and its membrane alignment and dynamics determined [5,6]. Structure analysis is straightforward for regular backbone folds, as for example α-helices or β-strands. The alignment of such secondary structure element in the lipid bilayer is then described in terms of its tilt (τ) and azimuthal rotation (ρ) angles, as illustrated in Figure 1 for the case of an α-helix. Additional dynamic information is extracted from the NMR data in terms of a molecular order parameter S_{mol}, which describes the motional averaging on a scale between 1.0 for an immobile system and zero for an isotropically tumbling molecule. Likewise, the

*Correspondence to: A. S. Ulrich, Institute of Organic Chemistry, Universität Karlsruhe (TH), Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany; e-mail: anne.ulrich@ibg.fzk.de

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analysis of macroscopically aligned samples can reveal whether a molecule undergoes fast long-axial rotation about the membrane normal on the time scale of the relevant NMR interaction. Such dynamic information can be interpreted in terms of peptide oligomerization or immobilization by aggregation. The membrane-bound peptide can therefore be comprehensively described by (i) its backbone conformation (α-helix, β-strand, etc.), (ii) its alignment in the lipid bilayer (τ, ρ) and (iii) its mobility (S_{mol}, long-axial rotation). These parameters, which are readily available from simple solid-state NMR experiments, can be monitored under different conditions and in different lipid environments. That way, details of the peptide–peptide and peptide–lipid interactions can be rationalized and mechanistic models can be suggested to explain biological function.

To label peptides for solid-state NMR analysis, traditionally the isotopes ^{13}C, ^{15}N and ^{2}H have been incorporated site-specifically by chemical synthesis, or uniformly by recombinant expression [2–4,7,8]. The major drawback of these labels, however, is their intrinsically low NMR sensitivity (^{15}N, ^{2}H) or natural abundance background (^{13}C). As a promising alternative, the use of ^{19}F has been explored in view of its much higher NMR sensitivity and lack of background signals [5,6,9,10]. Several different peptides have been successfully characterized by ^{19}F-NMR so far [11–16]. The ^{19}F-labelled amino acids used in these studies have to meet certain criteria, namely: (i) the reporter group has to be rigidly attached to the peptide backbone in order to reflect the global peptide structure, (ii) the labeled side chain must not perturb the local peptide conformation, (iii) an axially symmetric rotating CF_3-group is ideal for data analysis, and (iv) finally incorporation of the amino acid into a synthetic peptide should be straightforward by conventional Fmoc chemistry. Most previous studies have utilized 4-CF_3-phenylglycine (CF_3-Phg) (Figure 2(A)) as a substitute for aliphatic amino acids, which are abundant in membrane-active peptides. The only drawback of this amino acid is the fact that extensive racemization occurs under basic conditions, and hence the resulting epimeric peptide mixtures have to be carefully separated by HPLC. To circumvent this particular problem while maintaining all other advantages, we have rationally designed a novel amino acid with a CF_3-moity rigidly attached to the peptide backbone, namely 3-(trifluoromethyl)-bicyclopent- [1.1.1]-1-ylglycine (CF_3-Bpg, Figure 2(B)). This compound can be conveniently synthesized as a pure L-enantiomer on the gram-scale as recently reported [17]. We have tested the incorporation of CF_3-Bpg in various polypeptide sequences using Fmoc-based solid-phase peptide synthesis and have shown that no detectable racemization occurs under standard conditions, nor does any elimination occur or unforeseen problems arise. The peptides carrying a single CF_3-Bpg substitution in place of either Ala or Ile have shown virtually the same biological activity as the wild-type sequence and the same overall conformation as assessed by circular dichroism [17]. Hence, all necessary conditions are fulfilled now to apply this new label for ^{19}F-NMR structure analysis. In the present contribution we examine the utility, reliability and accuracy of CF_3-Bpg using the α-helical peptide PGLa as a scaffold for its incorporation. The

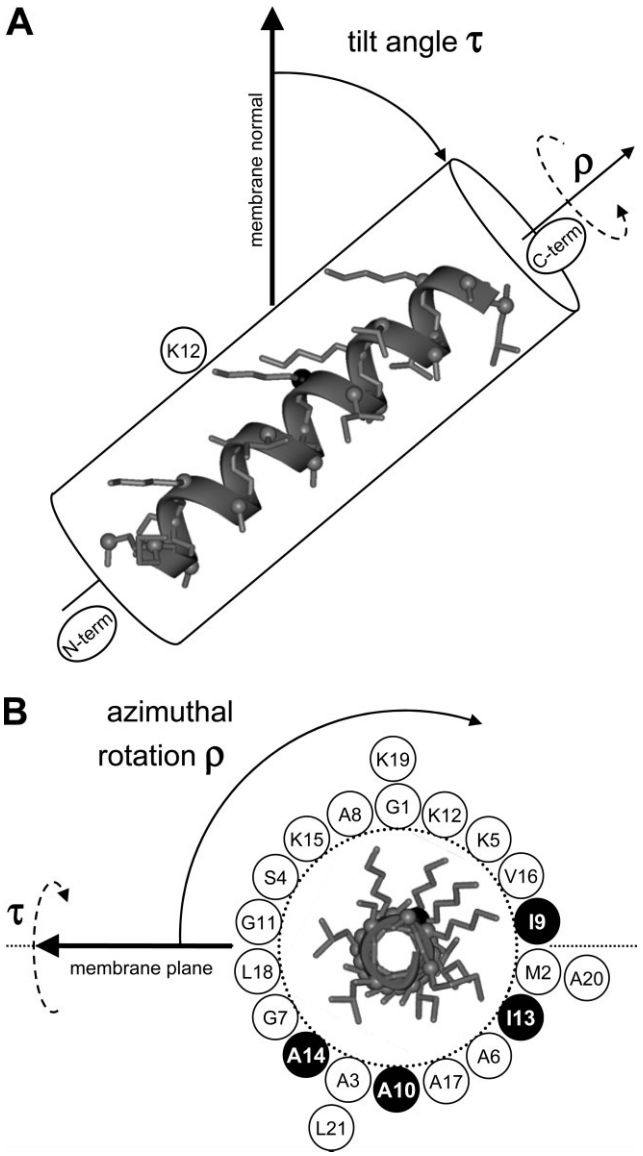

Figure 1 Structural parameters used to describe the alignment of an α-helical peptide in a lipid bilayer. (A) The tilt angle τ is defined between the membrane normal and the helix axis, with the *C*-terminus tilting away. (B) The helical wheel representation of PGLa illustrates the azimuthal rotation ρ around the helix axis, for which we define $\rho = 0°$ when Cα of Lys12 lies in the membrane plane. In (A) the molecule is positioned with $\tau = 50°$ and $\rho = 0°$, while in (B) $\tau = 90°$ and $\rho = 110°$. Amino acids substituted with NMR labels (Ile9, Ala10, Ile13 and Ala14) are highlighted, and Cα of Lys12 indicated by a black sphere.

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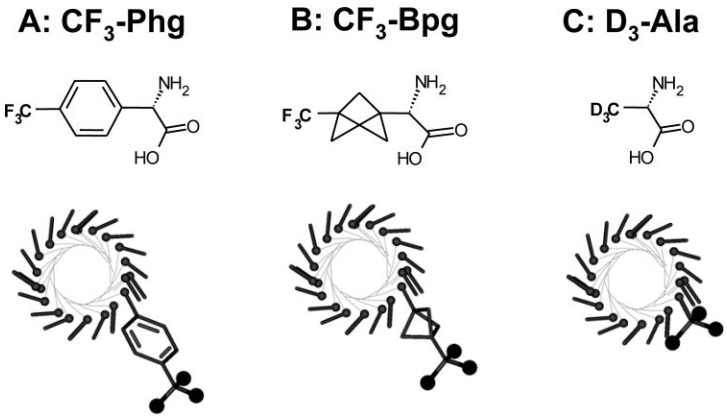

Figure 2 Side-chain labeled L-amino acids that are used as reporter groups for solid-state NMR, illustrated within the helical framework of PGLa. (A) 4-(CF_3)-phenylglycine; (B) 3-(CF_3)-bicyclopent-[1.1.1]-1-ylglycine; (C) alanine-3,3,3-d_3.

structural properties and dynamic behavior of this antimicrobial peptide are well known, as it has been thoroughly characterized using CF_3-Phg and 3,3,3-d_3-alanine (D_3-Ala) as labels (Figure 2). A considerable number of orientational constraints that are available from these solid-state ^{19}F- and ^{2}H-NMR studies can thus be compared with the new data of CF_3-Bpg [15,16,18–20].

The antimicrobial peptide PGLa (GMASKAGAIAGKIA KVALKAL-NH_2) from the skin of *Xenopus laevis* belongs to the magainin family of cationic amphiphilic membrane-active peptides [21,22]. These systems do not require a specific receptor but instead exert their antimicrobial function by disrupting the cell membranes of invading bacteria. In view of this physical mechanism of action, it is expected that bacteria will not be able to develop any resistance against this highly potent class of antibiotics [1,23–26]. To understand further details of their functional mechanism, the behavior of PGLa has been comprehensively characterized under a range of different conditions and in different lipid model membranes. The peptide is known to fold as an amphiphilic α-helix upon binding to a lipid bilayer, whereupon it lies flat on the surface in the so called 'S-state', which is characterized by a tilt angle τ (Figure 1) of about 90° [15,18,19]. In liquid crystalline dimyristoylphosphatidylcholine (DMPC) model membranes, the parameters of the S-state have been accurately described by solid-state ^{2}H-, ^{15}N- and ^{19}F-NMR ($\tau = 98°$, $\rho = 116°$, $S_{mol} = 0.71$, long-axial rotation occurring). With increasing peptide concentration, a realignment of PGLa was observed and a new, tilted 'T-State' was described with the same level of detail ($\tau = 126°$, $\rho = 113°$, $S_{mol} = 0.78$, long-axial rotation occurring) [16,18,19]. More recently, PGLa was shown to tilt even further to assume an inserted 'I-state' ($\tau \approx 160°$) in the presence of equimolar amounts of the synergistic antimicrobial peptide magainin 2. As the functional relevance of the S-, T- and I-states is beyond scope of this paper, it shall only be mentioned here that the former is monomeric, while the T-state is regarded as a homodimer and the I-state as a heterodimer [5,6,15,16,18–20].

MATERIALS AND METHODS

Amino Acid Synthesis and Incorporation into Peptides

CF_3-Bpg was synthesized and Fmoc-protected on a semipreparative scale as previously described [17]. The same article contains a detailed description of the incorporation of CF_3-Bpg into the synthetic peptides studied here. A single CF_3-Bpg label was introduced, one at a time, either at position Ile-9, Ala-10, Ile-13 or Ala-14 to substitute the corresponding native amino acid in the wild-type PGLa sequence. We will abbreviate these peptide analogs as PGLa-9-Bpg, PGLa-10-Bpg, PGLa-13-Bpg and PGLa-14-Bpg, respectively. The four labeled PGLa analogs were synthesized on an Applied Biosystems 431A peptide synthesizer using standard Fmoc protocols. They were cleaved from the resin with a TFA/H_2O/TIS cocktail, precipitated with diethyl ether, lyophilized and purified on a semipreparative scale by RP-HPLC (10 mm × 250 mm column). A C18 column was used with individually adjusted water/acetonitrile linear gradients, containing 5 mM HCl as an ion-paring agent (instead of TFA, which gives unwanted signals in ^{19}F-NMR). The identity and purity of the collected peptide fractions were evaluated by MALDI-TOF mass spectrometry and analytical RP-HPLC (4.6 mm × 250 mm column), confirming >95% purity.

Preparation of Macroscopically Aligned NMR Samples

All individually labeled PGLa analogs were reconstituted into DMPC (Avanti Polar Lipids, Alabaster, AL, USA) using two different peptide/lipid (P/L) molar ratios of either 1/50 or 1/200. Macroscopically aligned membrane samples were prepared on glass supports as previously described [5,6,27]. The dry peptide and lipid powders were codissolved in methanol/$CHCl_3$ (1/1, v/v) and deposited (30 µl/slide) on thin glass slides (0.08 × 7.5 × 18 mm^3, from Marienfeld Laboratory

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Glassware, Lauda-Königshofen, Germany). Either 10 glass slides (carrying 0.5 mg peptide in total, for P/L = 1/50) or 18 glass slides (carrying 0.25 mg peptide in total, for P/L = 1/200) were used to deposit around 1 mg total dry weight per slide. Once the slides appeared visually dry, they were further dried under vacuum overnight to remove all traces of the solvent. The slides were then manually stacked and placed into a closed container with an atmosphere of 96% relative humidity at 48 °C for 24 h. This hydration procedure results in an uptake of water by the peptide–lipid multilayers and their spontaneous alignment parallel to the glass surface. The samples were thereafter wrapped with parafilm and polyethylene foil to maintain the hydration during the NMR measurement. When the samples were not in use, they were typically stored at −20 °C. The degree of orientation and level of hydration in the samples were checked by ^{31}P-NMR before and after the ^{19}F-NMR experiments. If resonances from the oriented lipids were broadened, the material was considered to have partially dried out, hence it was rehydrated and remeasured. Rehydration was achieved by removing the wrapping, dipping the stack into pure H_2O for 15 s and subsequently placing the stack in the hydration chamber for 12 h.

NMR Spectroscopy

All solid-state NMR measurements were carried out on a Bruker Avance 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) at 35 °C. The real temperature in different probeheads was calibrated using a methanol chemical shift thermometer as suggested in Ref. 28. ^{31}P-NMR experiments were performed at 202 MHz using a standard Bruker triple-resonance, wideline probe equipped with a flat solenoid coil. A Hahn echo sequence with a 90° pulse length of 6 μs and an echo delay time of 30 μs was used to acquire ^{1}H-decoupled ^{31}P-NMR spectra of oriented membranes. ^{19}F-NMR was performed at 470 MHz with a ^{19}F/^{1}H double-tuned, flat-coil probe (Doty Scientific, Columbia, SC, USA) that could be manually tilted. Simple 1-pulse experiments with a 90° pulse width of 1.8 μs and 10–15 kHz TPPM ^{1}H-decoupling were used to acquire regular ^{19}F-NMR spectra. ^{19}F spectra were referenced by setting the fluoride resonance of 100 mM NaF aqueous solution (observed at 35 °C) to −119.5 ppm [14].

Structure Calculation

The homonuclear ^{19}F–^{19}F dipolar couplings of CF_3-Bpg labels in four different positions of PGLa were collected and used to calculate the orientation of the peptide in the lipid bilayer. The backbone was modeled as an ideal α-helix, and the geometry of the labeled side chain was described by two parameters $\alpha = 121.1°$ (angle between $C_\alpha - C_\beta$ and the helix long axis Z) and $\beta = 53.2°$ (angle between the projection of the $C_\alpha - C_\beta$ bond onto the X-Y plane and the radial axis) for D_3-Ala, while $\alpha = 110°$ and $\beta = 47.0°$ were used for the bulky CF_3-Bpg and CF_3-Phg side chains [15]. The alignment of this ideal model structure in the lipid bilayer is then described by a tilt angle τ with respect to the membrane normal, and by an azimuthal rotation ρ around the helix axis (Figure 1). To determine these two values together with the order parameter S_{mol}, all three parameters were systematically varied in a grid search, and a least-squares fit was performed to find the globally smallest root mean square deviation (RMSD) between the experimental and calculated NMR parameters. The method is described in detail in a number of recent reviews [1,5,6].

RESULTS AND DISCUSSION

Choice of Conditions to Characterize PGLa

Macroscopically oriented DMPC model membranes containing PGLa at two different peptide–lipid ratios (1/200 and 1/50) were prepared and examined at 35 °C, for the following reasons. First of all, ^{19}F-NMR is sensitive enough to study oriented samples even at very low peptide concentration [14]; hence this sample geometry was used to provide excellent spectral resolution and to check for rotation of the peptide around the membrane normal. Furthermore, in oriented DMPC multibilayers the binding equilibrium of PGLa is driven towards complete binding owing to the absence of bulk water [19]. Indeed, the cationic peptide was shown to have the same α-helical structure in DMPC as in charged DMPC/DMPG mixtures [15,16,18–20], even though in solution it prefers negatively charged liposomes or detergent micelles [15,17,29]. In view of some peculiar phase properties of DMPC/DMPG [30,31], we therefore decided to keep the peptide–lipid system as simple as possible. In the liquid crystalline state of DMPC (>24 °C), PGLa spontaneously adopts the S-state at low peptide concentration (P/L ≤ 1/200). Upon increasing the peptide concentration (P/L ≥ 1/50) a realignment of PGLa into a tilted T-state has been described, in which the overall helical fold and amphiphilic mode of binding are retained [16,18,19]. The exact threshold concentration for realignment in oriented DMPC bilayers was found to be at P/L = 1/80 by oriented circular dichroism (unpublished results), and in this concentration regime a fast interconversion between the two states was reported by ^{2}H-NMR [19]. Therefore, to characterize PGLa under conditions of a pure S-state and a pure T-state, two discrete peptide–lipid ratios of 1/200 and 1/50 in DMPC are selected here. Higher concentrations (P/L ≥ 1/35) may lead to an immobilization of the peptides [16]. To evaluate the four labelled peptides PGLa-9-Bpg, PGLa-10-Bpg, PGLa-13-Bpg and PGLa-14-Bpg, we have therefore prepared eight samples altogether (4 × 2 concentrations), providing one set of data each for the expected S-state (P/L = 1/200) and T-State (P/L = 1/50). To monitor the state of the lipid bilayers in the oriented samples, ^{31}P-NMR spectra were acquired before and after the ^{19}F-NMR measurements (data not shown). The lineshapes revealed well-oriented lipid molecules in a liquid crystalline lamellar phase, in which the observed degree of orientation (70–80%) is typical for a high-quality sample of this kind. No systematic differences were seen between low and high concentration, and the samples remained stable during the ^{19}F-NMR measurements (typically 1 h).

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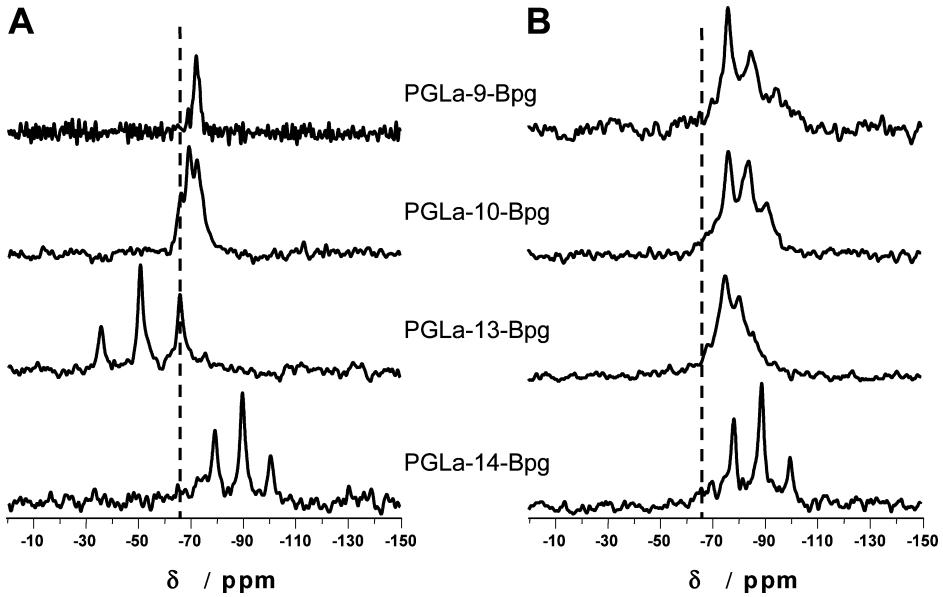

Figure 3 Solid-state ^{19}F-NMR spectra of four PGLa analogs labeled with CF_3-Bpg at different positions (Ile9, Ala10, Ile13, or Ala14), reconstituted into macroscopically oriented DMPC bilayers. Two peptide/lipid ratios are compared here, namely (A) 1/200 corresponding to the surface-bound S-state, and (B) 1/50 corresponding to the tilted T-state. All samples were measured at 35 °C, with the membrane normal parallel to the magnetic field.

Solid-State ^{19}F-NMR Spectra

Figure 3 shows the ^{19}F-NMR spectra from eight oriented samples containing PGLa labeled with CF_3-Bpg in four different positions (Ile9, Ala10, Ile13, Ala14), embedded in DMPC bilayers at a peptide–lipid ratio of 1/200 and 1/50. All peptides are well aligned, as there are no powder lineshapes seen, and the individually labelled positions obviously give rise to different spectra. Differences are also seen for the two peptide–lipid ratios, suggesting that the alignment of PGLa indeed differs in the two concentration regimes addressed here. As expected, the ^{19}F-NMR signals of the CF_3-labels appear as triplets with an isotropic chemical shift at -66 ppm, covering a spectral range (−30 to −110 ppm), which is typical for CF_3-moieties of organic compounds. The ^{19}F–^{19}F dipolar splittings (measured as the distance between two adjacent lines) vary from zero to 7.5 kHz, and the full widths at half-height of the central peak are about 1 kHz (P/L of 1/200) to 2.5 kHz (P/L of 1/50). In Figure 3 the samples were measured with their normal parallel to the magnetic field, but when they were manually rotated by 90° all splittings were reduced by a factor of 2. This observation shows that PGLa undergoes fast long-axial rotation about the membrane normal in liquid crystalline DMPC. At this stage we may point out the intrinsic advantage of ^{19}F-NMR in terms of sensitivity, as the present spectra were recorded in about 1 h using only 0.25–0.5 mg of peptide.

The spectra of CF_3-Bpg can now be compared with our previous ^{19}F-NMR data of PGLa acquired from four CF_3-Phg labels attached to the very same positions in the peptide [15,16,18–20]. Both the range of dipolar splittings and their signs are similar in both cases, as summarized in Table 1, suggesting a comparable structural behavior of the peptide in both labeling schemes. Interestingly, deviations within any individual triplet from the ideal 1 : 2 : 1 intensity ratio are much less pronounced in the case of CF_3-Bpg than for CF_3-Phg, presumably as a result of motionally induced T_2-relaxation (as discussed in [15]). This observation and the fact that the CF_3-Bpg spectra exhibit much better resolved triplets, suggest that the new label

Table 1 Experimental ^{19}F–^{19}F dipolar splittings of the CF_3-Bpg and CF_3-Phg in PGLa, reconstituted in oriented DMPC bilayers at different peptide/lipid ratios

Labeled position	Dipolar splitting/kHz	
	CF_3-Bpg	CF_3-Phg[a]
	P/L = 1/200 (S-state)	
Ile9	0.0	0.0
Ala10	−1.4	0.0
Ile13	7.5	5.6
Ala14	−4.9	−5.4
	P/L = 1/50 (T-state)	
Ile9	−4.5	−4.7
Ala10	−3.0	−3.1
Ile13	−3.4	−3.3
Ala14	−5.4	−5.3

[a] Values taken from [15,16].

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Table 2 Best-fit solutions for the structure and dynamics of PGLa in DMPC, obtained using different solid-state NMR Labels. The number of constraints used and their positions in PGLa are indicated. (τ) – Helix tilt angle; (ρ) – Azimuthal rotation angle; S_{mol} – Order parameter; (RMSD) – Root mean square deviation between the experimental and calculated values of the spectral splittings

Labels used	Positions	τ [°]	ρ [°]	S_{mol}	RMSD [kHz]
		P/L = 1/200 (S-state)			
4x CF_3-Bpg	I9, A10, I13, A14	98	116	0.68	0.2
4x CF_3-Phg	I9, A10, I13, A14	97	112	0.60	0.5
4x D_3-Ala	A6, A8, A10, A14	98	116	0.66	0.2
8x D_3-Ala	A6, G7, A8, I9, A10, G11, I13, A14	98	116	0.71	0.4
		P/L = 1/50 (T-state)			
4x CF_3-Bpg	I9, A10, I13, A14	134	89	0.63	0.3
4x CF_3-Phg	I9, A10, I13, A14	134	91	0.63	0.2
4x D_3-Ala	A6, A8, A10, A14	126	111	0.75	0.08
8x D_3-Ala	A6, G7, A8, I9, A10, G11, I13, A14	126	113	0.78	0.2

may have intrinsically better properties for ^{19}F-NMR structure analysis of peptides.

Alignment of PGLa Calculated from the CF_3-Bpg Labels

Having collected four sets of orientational constraints for each peptide–lipid ratio, we are now able to calculate the structure and (re)alignment of PGLa from the CF_3-Bpg labels. The main objective of this work is to compare the analytical solution derived from the new orientational constraints with previous results from CF_3-Phg and nonperturbing D_3-Ala labels. Any perturbation of the peptide conformation by CF_3-Bpg or any unforeseen problems in its analysis should therefore become apparent in a critical evaluation. In the following discussion of Figure 4 (P/L = 1/200) and Figure 5 (P/L = 1/50) we will first compare the raw NMR data in panel (A), before describing the peptide structure in terms of the corresponding best-fit solutions for τ and ρ. In both figures our new results from the four CF_3-Bpg labels in positions Ile9, Ala10, Ile13 and Ala14 are displayed in panel (B), the previous results from four CF_3-Phg labels in the same positions are displayed in panel (C), the results from four entirely nonperturbing D_3-Ala labels (positions Ala6, Ala8, Ala10, Ala14) in panel (D) and the results from eight D_3-Ala labels (positions Ala6, Gly7, Ala8, Ile9, Ala10, Gly11, Ile13, Ala14) in panel (E). All corresponding values of τ and ρ are summarized in Table 2 together with the best-fit values of S_{mol}, and in all cases the peptides were found to rotate about the membrane normal.

There are two convenient ways by which the analytical results on the PGLa structure can be graphically illustrated and errors examined. On the one hand, the best-fit solution from a grid-search over τ, ρ and S_{mol} can be used to construct a dipolar wave. Here, the expected splitting for any position in an ideal helical wheel is plotted as a function of the sequence and displayed between 0 and 360°. Any deviation of an individual experimental data point from the idealized α-helical wave can thus be recognized and interpreted accordingly. Figures 4(A) and 5(A) display all experimental data from the current and previous ^{19}F- and ^{2}H-NMR analysis of PGLa labeled with CF_3-Bpg, CF_3-Phg and D_3-Ala. To be able to compare the ^{2}H-NMR quadrupole splittings directly with the ^{19}F-NMR dipole splittings, we used a factor of 5.3 to scale down all values from the D_3-Ala labels [18]. This proportionality factor is the ratio between the maximum Pake splitting in the powder spectrum of a rotating but otherwise immobilized CD_3-group (84 kHz [32]) and that of a CF_3-group (15.8 kHz [15]). It is seen that the dipolar waves look very similar within any one panel, but they differ significantly for the two peptide–lipid ratios. A slight shift in the curves of the ^{19}F-NMR data (4x CF_3-Bpg, 4x CF_3-Phg) *versus* the ^{2}H-NMR data (4x D_3-Ala, 8x D_3-Ala) is noted, especially at 1/50, which will be discussed below. We can nevertheless conclude that all labeling schemes yield the same consistent picture of PGLa, namely an unperturbed α-helix, which assumes two distinct orientations in the membrane at high and low peptide concentration.

An alternative representation of the structural results makes use of the τ/ρ maps. The contour levels show the RMSD between experimental and calculated data for all possible combinations of τ and ρ, which are systematically sampled in a grid search. The analysis is repeated by varying S_{mol} stepwise, and the best-fit solution is displayed as the corresponding RMSD surface. If several deep minima are present, the number of data points is not sufficient to find a unique solution and further input is required. It is evident from Figures 4 and 5 that the new CF_3-Bpg labels

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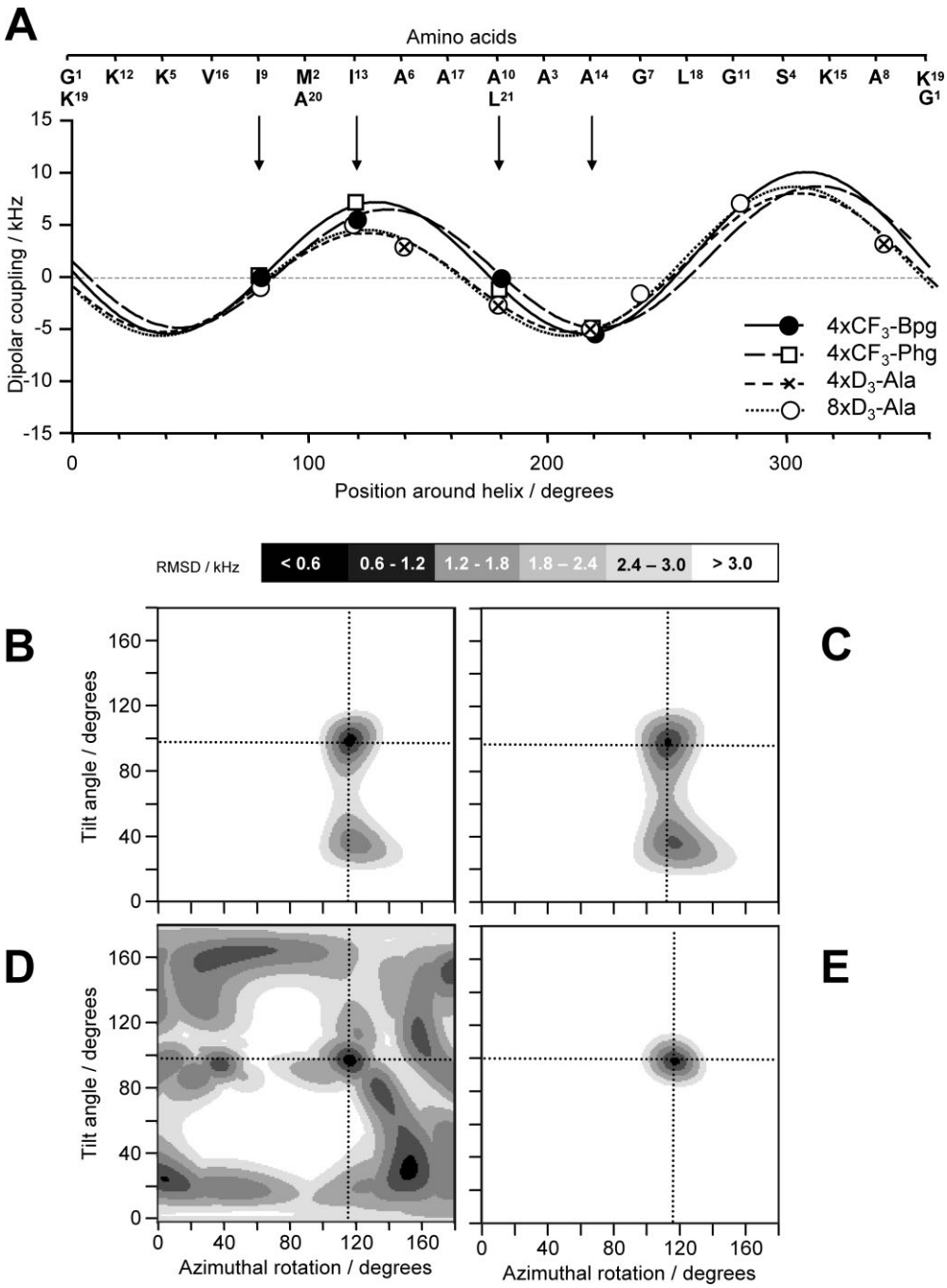

Figure 4 Structure analysis of PGLa at a peptide/lipid ratio of 1/200, corresponding to the surface-bound S-state. (A) The dipolar wave plot shows the experimental splittings and best-fit solutions obtained from: four CF_3-Bpg labels (solid line, filled circles); four CF_3-Phg labels (dashed line, open squares); four nonperturbing D_3-Ala labels (short dashed line, crosses); and altogether eight D_3-Ala labels (dotted line, open circles). The ^{2}H-NMR quadrupole splittings have been translated to the dipolar scale of ^{19}F-NMR (see text), and the amino acid sequence is indicated above, arrows denoting the positions of the CF_3-Bpg labels used in this study. (B–E) Error plots showing the best-fit values of τ and ρ (as well as S_{mol}, not indicated here, but see Table 2) from: (B) 4x CF_3-Bpg; (C) 4x CF_3-Phg; (D) 4x D_3-Ala; and (E) 8x D_3-Ala.

(panels B) yield the same solution as the old CF_3-Phg data (panels C) within error limits. The global RMSD minimum occurs in the same position of the τ/ρ map, as confirmed by the values of τ, ρ and S_{mol} in Table 2, yet these results differ significantly for the two peptide–lipid ratios. The increase in helix tilt angle by over 30° in going from low to high concentration confirms that the CF_3-Bpg labeled PGLa realigns from the S-state to the T-state as expected.

Turning to the ^{2}H-NMR data of Figures 4 and 5, it is striking to see that four D_3-Ala labels generate far more local minima in the τ/ρ maps (panels D) than

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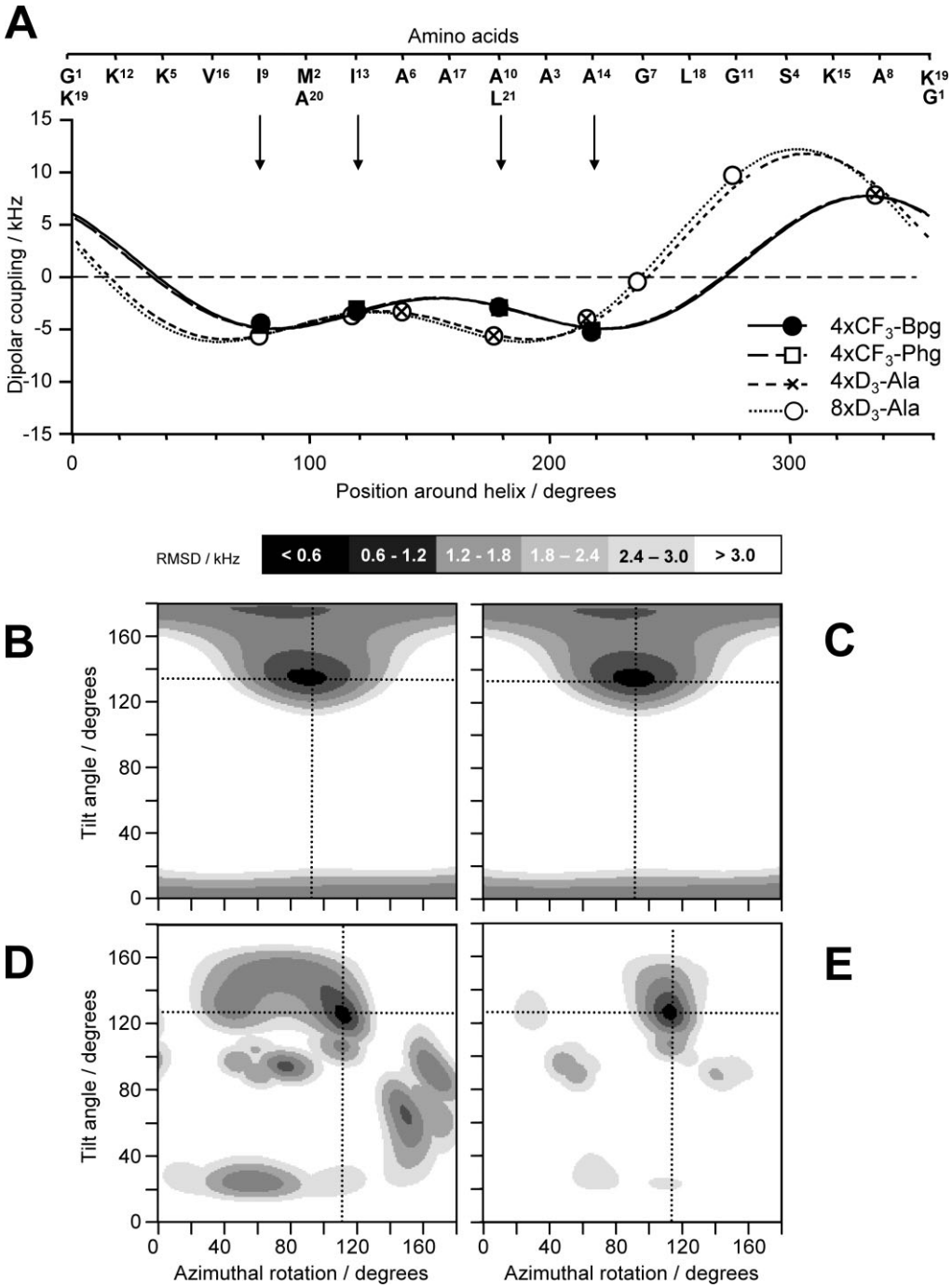

Figure 5 Structure analysis of PGLa at a peptide/lipid ratio of 1/50, corresponding to the tilted T-state. Remaining legend as in Figure 4.

the same number of CF_3-labels (panels B and C). For the initial ^{2}H-NMR analysis only the four native alanine positions of PGLa had been labeled to avoid any potential perturbation of the peptide, but this obviously resulted in an ill-defined solution. The reason for the multiple minima lies in the fact that positive and negative signs of deuterium quadrupole splittings cannot be distinguished from the symmetric ^{2}H-NMR spectra; hence four CD_3-labels are not sufficient to deduce the helix alignment in terms of τ, ρ and S_{mol} [18,19]. In contrast, the ^{19}F-NMR spectrum of a CF_3-group (Figure 3) displays not only the dipolar splitting but its sign is simultaneously revealed by the chemical shift anisotropy, which moves the triplet downfield or upfield for positive and negative signs, respectively [15,16]. To obtain a unique solution by ^{2}H-NMR, a total of eight D_3-Ala labels finally had to be used, as illustrated in Figures 4(E) and 5(E). Notable, even though four potentially perturbing sites (Ile, Gly) were substituted in the latter analysis besides the four native Ala

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positions, this did not lead to any significant deviation in the resulting structure (Figures 4(A) and 5(A)).

All results on PGLa are summarized in Table 2, which shows that essentially the same values for τ, ρ and S_{mol} are obtained at low peptide concentration (P/L = 1/200) for the surface-bound state, irrespective of which NMR labels are analyzed. The helix tilt angle in the S-state is close to 98°, and the azimuthal rotation of about 116° is in perfect agreement with the expected orientation of the charged residues towards the aqueous phase (Figure 1). Upon increasing the peptide concentration to 1/50, the change in tilt angle by over 30° demonstrates that all PGLa analogs are flipped into the T-state (in which the azimuthal angle is roughly maintained). A closer look reveals some small systematic differences between the ^{19}F- and ^{2}H-NMR results, as noted before in the wave plots of Figure 5(A) and to a lesser extent Figure 4(A). Interestingly CF_3-Bpg behaves very similar to CF_3-Phg, but both differ slightly from D_3-Ala with regard to the tilt angle (about 8°) and the azimuthal rotation (about 20°). This observation might be explained by the properties of the two types of side chains (Figure 2); namely, D_3-Ala is small and compact, and the geometry of its $C_\alpha - C_\beta$ bond is well known from many previous studies. On the other hand, the bulky side chains of CF_3-Bpg and CF_3-Phg are expected to differ from Ala, both in terms of their $C_\alpha - C_\beta$ orientation and in the local vibrations (which will affect the order parameter). The observed deviations therefore suggest that the specific geometric parameters we have used to model the respective side chains may not yet be optimal [15,33]. However, it does not appear feasible to refine the parameters for the $C_\alpha - C_\beta$ bonds from the present NMR data, given that an intrinsic error range of 10° for τ and 15° for ρ is associated with an uncertainty of 1 kHz in the experimentally determined splittings.

CONCLUSIONS

We have demonstrated that the novel amino acid CF_3-Bpg is a reliable label for the structure analysis of membrane-bound peptides by solid-state ^{19}F-NMR. It can be readily incorporated into synthetic peptides without racemization or elimination, to yield orientational constraints. Here, four individual labels in the α-helical framework of PGLa were sufficient to determine the alignment and dynamic behavior of this antimicrobial peptide in DMPC membranes. The structures observed for the S-state and the T-state confirm that the CF_3-Bpg results are fully consistent with earlier high-fidelity data from nonperturbing D_3-Ala labels and with a previous analysis based on CF_3-Phg. We may therefore conclude that substitution of a single aliphatic amino acid by another one (e.g. Ile/Ala → CF_3-Bpg, Ile/Ala → CF_3-Phg, Ile/Gly → D_3-Ala) does not cause any significant structural perturbations in PGLa, even though the Cα–Cβ geometry may deviate slightly from case to case. Given the fact that the signed dipole splittings of four CF_3-labels yield about as much information as the unsigned quadrupole splittings of eight CD_3-labels, it appears worthwhile to pursue the ^{19}F-labeling strategy.

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