Solution and Solid-State Nuclear Magnetic Resonance Structural Investigations of the Antimicrobial Designer Peptide GL13K in Membranes

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Supporting Information

ABSTRACT: The antimicrobial peptide GL13K encompasses 13 amino acid residues and has been designed and optimized from the salivary protein BPIFA2 to exhibit potent bacteriocidal and anti-biofilm activity against Gram-negative and Gram-positive bacteria as well as anti-lipopolysaccharide activity *in vitro* and *in vivo*. Here, the peptide was analyzed in a variety of membrane environments by circular dichroism spectroscopy and by high-resolution multidimensional solution nuclear magnetic resonance (NMR) spectroscopy. Whereas in the absence of membranes a random coil conformation predominates, the peptide adopts a helical structure from residue 5 to 11 in the presence of dodecylphosphocholine micelles. In contrast, a predominantly β -sheet structure was observed in the presence of lipid bilayers carrying negatively charged phospholipids. Whereas ¹⁵N solid-state NMR spectra are indicative of a partial alignment of the peptide ¹⁵N–¹H vector along the membrane surface, ²H and ³¹P solid-state NMR spectra indicate that in this configuration the peptide exhibits pronounced disordering activities on the phospholipid membrane, which is possibly related to antimicrobial action. GL13K, thus, undergoes a number of conformational transitions, including a random coil state in solution, a helical structure upon dilution at the surface of zwitterionic membranes, and β -sheet conformations at high peptide:lipid ratios.

Antimicrobial peptides and proteins (AMPs) are found in vertebrates, invertebrates, plants, and bacteria, and several of these naturally occurring AMPs have inspired the design of novel AMPs.^{1,2} Synthetic peptide design aims to optimize strong antibacterial activity while avoiding bacterial resistance or mammalian toxicity. AMP sequences are highly diverse, but certain principles of their design have emerged. Thus, short AMPs appear to require a balance of charged (R and K) and hydrophobic amino acids (I, V, F, Y, and W).³ We have designed the peptide GL13K by analysis of the salivary protein BPIFA2 (BPI-fold family A member 2; previously named parotid secretory protein, PSP, SPLUNC2, and C20orf70), a potential host-defense protein. The predicted structure of BPIFA2 was compared with the known structures of related BPI proteins⁴ and the location of AMPs within the sequences of these proteins.⁵ An initial peptide, GL13NH2

(BPIFA2_{141–153}), caused bacterial agglutination but did not exhibit bacteriocidal activity.⁶ The distribution of cationic and hydrophobic residues was rebalanced by substituting charged residues with Lys residues, resulting in the peptide GL13K, which is bacteriocidal to Gram-negative and Gram-positive bacteria and their biofilms.^{6,7} Both GL13NH2 and GL13K also exhibit anti-lipopolysaccharide activity *in vitro* and *in vivo*.⁶

Initial membrane interaction studies have shown that GL13K is highly selective for anionic bacterial model membranes (DOPG) compared to zwitterionic (neutral) eukaryotic model membranes (DOPC). In the presence of DOPC, the peptide exhibits a random coil conformation in circular dichroism (CD)

Received: June 2, 2017

Revised: July 6, 2017

Published: July 12, 2017

spectroscopic investigations and adopts a β -sheet structure in the presence of DOPG.⁸ Thus, as with other cationic amphipathic peptides, the extent of association with overall neutral membranes is probably too low to be detected by optical spectroscopy, which, due to light scattering, is limited to dilute solutions of small vesicles.

Here we use multidimensional solution NMR spectroscopy to investigate the high-resolution structure of GL13K in the presence of dodecylphosphocholine (DPC) micelles, thus, representing its structure when it is associated with zwitterionic membranes at low P:L ratios (on average fewer than one peptide is associated with each micelle). Furthermore, a bacterial membrane was mimicked by 3:1 (molar ratio) POPE/POPG model bilayers. The peptide's secondary structure preference at this intermediate membrane lipid composition with only 25 mol % negatively charged lipids was first determined by CD spectroscopy. Thereafter, the peptide topology and its interactions with lipids were investigated by reconstituting GL13K, labeled with ²H and ¹⁵N at specific sites, into bilayers aligned along planar glass plates or in bicelles that spontaneously align in the magnetic field of the NMR spectrometer, and measurement of the corresponding solid-state NMR spectra. Finally, the effect of the peptide on membrane packing was investigated by ²H solid-state NMR of fatty acyl chain deuterated lipids. In this manner, a more comprehensive view of the interactions of GL13K with membranes is obtained covering low and high peptide:lipid ratios as they occur with overall neutral, zwitterionic, and negatively charged bacterial membranes.

MATERIALS AND METHODS

Materials. Amino acid derivatives and other reagents for peptide synthesis were from Novabiochem-Merck (Darmstadt, Germany). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Water (HPLC grade) and deuterium-depleted water were from Sigma (St. Quentin Fallavier, France). Deuterated dodecylphosphocholine was purchased from Cambridge Isotope Laboratories (Andover, MA).

Peptide Synthesis. The GL13K peptide (GKIIK**L**KASL-KLL-NH₂) was obtained by solid phase peptide synthesis using a Millipore 9050 automated peptide synthesizer and Fmoc (9-fluorenylmethyloxycarbonyl) chemistry. The peptide was purified by semipreparative HPLC as described previously for other antimicrobial peptides.⁹ The underlined leucine 6 and the bold alanine 8 positions were labeled with ¹⁵N and ²H₃, respectively, by incorporation of the isotopically labeled fmoc precursors (Euriso-top, Paris, France, or Isotec Sigma-Aldrich, St. Quentin Fallavier, France). The identity and high purity of the product (>90%) were confirmed by HPLC, MALDI mass spectrometry, and NMR spectroscopy. After lyophilization, the TFA counterions were exchanged in 5% (v/v) acetate. The randomized peptide GL13K-R1 with the sequence IGKLLK-SKLKAL-NH₂ was obtained from AAPPTec (Louisville, KY).

Micellar Sample Preparation and Solution NMR Spectroscopy. The lyophilized peptide was dissolved to a final concentration of 1.9 mM in an aqueous solution (10% D₂O and 90% H₂O) containing 186 mM perdeuterated DPC (Cambridge Isotope Laboratories) and 10 mM phosphate buffer at pH 5. All the NMR experiments were performed at 298 K on a Bruker 700 MHz spectrometer (Bruker Biospin, Billerica, MA). The homonuclear two-dimensional (2D) TOCSY¹⁰ (70 ms) and 2D NOESY¹¹ (100–250 ms mixing time) experiments were performed in phase-sensitive mode

using time-proportional phase incrementation. A WATERGATE pulse sequence¹² was used for solvent suppression. During the isotropic mixing time, a DIPSI-2 pulse sequence¹³ was used in the TOCSY experiments. In all experiments, the spectral width was 8000 Hz in both dimensions. 2D data were zero filled to 4096 points in the direct dimension and to 1024 points in the indirect dimension and then processed using a sine-bell squared window function shifted between 60° and 90° before Fourier transformation. For baseline correction, a sixth-order polynomial function was used.

All the spectra were processed using NMRPipe,¹⁴ and the data were analyzed using Sparky.¹⁵ The traditional resonance assignment approach described by Wüthrich¹⁶ was applied.

Structure Calculation. A total of 151 NOEs, including 70 intra-residue and 81 inter-residue NOEs, were used for structure calculation. NOE cross-peaks from 100–250 ms mixing time ¹H–¹H NOESY experiments were integrated and classified as strong, medium, and weak corresponding to distance restraints of 1.8–2.9, 1.8–3.6, and 1.8–6.0 Å, respectively. The GL13K structural ensemble was calculated starting from the extended structure and minimized using a hybrid simulating annealing (SA) protocol present in XPLOR-NIH.¹⁷ One hundred conformers were generated at an initial temperature of 1000 K with 50000 high-temperature steps and 6000 cooling steps. Structural refinement was performed by introducing Lennard-Jones potentials and van der Waals and electrostatic interactions using an initial temperature (*T*_i) of 1000 K with 10000 cooling steps with a 5 fs step size. The final refinement of the structural ensemble was calculated at a *T*_i of 600 K with 50000 cooling steps with a 1 fs step size.

Circular Dichroism Spectroscopy. POPE/POPG (3:1 molar ratio) and POPC/POPS (3:1 molar ratio) lipids were mixed in chloroform, dried under a stream of nitrogen gas, kept under vacuum overnight, rehydrated with 5 or 10 mM Tris-HCl at pH 7.4, and subjected to three freeze/thaw cycles. Small unilamellar vesicles (SUVs) were obtained after tip sonication with a Bandelin Sonopuls HD 200 (Berlin, Germany) for <1 min. Circular dichroism (CD) spectra were recorded from 250 to 190 nm (spectral resolution of 1 nm, data pitch of 1 nm, scanning speed of 50 nm/min) using a Jasco J-810 or a J-815 spectropolarimeter (Jasco, Tokyo, Japan) and a quartz cell with a 1.0 mm path length.

For lipid titration experiments, the samples containing 60 μ M GL13K peptide in 5 mM Tris-HCl buffer (pH 7.4) were maintained at 37 °C for titration with POPE/POPG SUVs and at 25 °C for titration with POPC/POPS SUVs. A 13.6 mM stock solution of SUVs was used for the peptide solution titration at different peptide:lipid ratios. A total of nine spectra were acquired and averaged for each condition. A mock titration of lipids into 5 mM Tris-HCl without peptide was used to generate control curves, which were subtracted from the CD spectra of peptide-containing samples. The resulting intensities in millidegrees were converted to mean residue molar ellipticity ($MRE = \theta/10nCl$, where *n* is the number of peptide bonds, *C* the concentration in molar, and *l* the path length in centimeters).

To obtain the pH-dependent circular dichroism (CD) spectra of GL13K and GL13K-R1, the peptides were dissolved, at a concentration of 0.1 mM, in a series of sodium borate buffers at pH 8.5, 9, 9.2, 9.4, 9.6, 9.8, 10, 10.2, 10.4, and 10.6. CD spectra were acquired with solvent subtraction. To examine the percentage of secondary structure of peptides, the spectra were fitted using three different programs (CDSSTR, 185

CONTINLL, and SELCON3) in CD Pro,¹⁸ and the averaged percentage is reported. Alternatively, an in-house program was used to fit the experimental spectra by finding optimal linear combinations of published peptide spectra.

Sample Preparation for Mechanically Oriented Membranes. A homogeneous mixture of POPE and POPG (3:1 molar ratio) and [¹⁵N-L6,²H₃-A8]GL13K was obtained by co-dissolving the components in chloroform and methanol [1:1 (v:v)]. The solution was spread onto ultrathin cover glasses (8 mm × 22 mm, Marienfeld, Lauda-Königshofen, Germany) and dried, first in air and thereafter under high vacuum overnight. Membranes were equilibrated at 93% relative humidity, and in cases in which it was necessary to obtain hydration in the liquid-disordered state, the membranes were further exposed to a higher temperature (310 K) before the glass slides were stacked on top of each other and sealed in Teflon and then in a plastic wrapping.

Sample Preparation for Magnetically Aligned Bicelles. [¹⁵N-L6,²H₃-A8]GL13K (2.5 mg) was added to a solution of 131 mM 1,2-dihexanoyl-*sn*-glycero-3-phosphocoline (DHPC). This solution was added to the long chain lipids DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocoline) and DMPG [1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)] in a 4:1 DMPC:DMPG molar ratio. The lipid composition was arranged so that the DMPC:DMPG/DHPC sample has a lipid concentration of 28% (w:v) and a molar ratio q ([DMPC:DMPG]/[DHPC]) of 3.5. To mimic physiological conditions, a suitable volume of 100 mM NaCl in water at pH 7.4 was added. Thus, the sample contained 78 wt % water for all preparations (h = mass of water/total mass of phospholipids and water). Hydrated samples were precipitated in a model 5804R Eppendorf benchtop centrifuge with a model F-45-30-11 rotor (Eppendorf AG, Hamburg, Germany) at 6500 rpm (~4500g) for 5 min and vigorously vortexed. The samples were then frozen in liquid nitrogen for 30 s, heated at 40 °C for 10 min in a water bath, vigorously vortexed, and centrifuged again at 6500 rpm for 5 min at 4 °C. This cycle was repeated three times until a homogeneous suspension (viscous and translucent) was obtained at room temperature.

Solid-State NMR Spectroscopy. NMR experiments were performed on Bruker Avance spectrometers operating either at 11.7 T (¹H frequency of 500 MHz) or at 17.7 T (750 MHz). ³¹P NMR spectra were recorded at 202.4 MHz (11.7 T) using a phase-cycled Hahn echo pulse sequence with gated broadband proton decoupling.¹⁹ Typical acquisition parameters were as follows: spectral window, $\pi/2$ pulse width, and recycle delay of 100 kHz, 6 μ s, and 5 s, respectively, for mechanically aligned samples and 20 kHz, 7.1 μ s, and 5 s, respectively, for aligned bicelles. Typically, 1024 scans were recorded. A line broadening of 50 Hz was usually applied prior to Fourier transformation. Quadrature detection was used in all cases. Phosphorus chemical shifts were calibrated relative to H₃PO₄ (85% in H₂O, 0 ppm). Samples were allowed to equilibrate for at least 20 min at (40 ± 1) °C before the NMR signal was acquired.

²H NMR spectra were recorded at 76.8 MHz by means of a quadrupolar echo pulse sequence,²⁰ with a $\pi/2$ pulse width of 7.5 μ s and a 50 μ s interpulse delay for mechanically oriented samples (4.5 and 100 μ s, respectively, for bicelles) and a recycle delay of 1 s. Typically, 20000 scans were recorded, and a Lorentzian noise filtering width of 200 Hz was applied prior to Fourier transformation from the top of the echo signal. Quadrature detection was used in all cases. The reference for

solid-state deuterium powder patterns was set to zero and the position of the carrier placed in the middle of the symmetric Pake pattern (powder spectrum). Samples were allowed to equilibrate for at least 20 min at (40 ± 1) °C before the NMR signal was acquired. The S_{CD} order parameters in bilayer membranes are proportional to the quadrupolar splittings:^{21,22} $S_{CD} = 4\Delta\nu_Q/3A_Q$, where A_Q is 167 kHz.

Proton-decoupled ¹⁵N cross-polarization (CP) spectra were acquired at 50.7 MHz (11.7 T) for mechanically aligned samples and at 76.8 MHz (17.7 T) for bicelles. A CP pulse sequence was used, with a spectral width, acquisition time, CP contact time, $\pi/2$ pulse width, and recycle delay of 50 kHz, 10 ms, 0.8 ms, 10 μ s, and 3 s, respectively, for mechanically oriented samples and 50 kHz, 10 ms, 0.4 ms, 8 μ s, and 2 s for magnetically oriented bicelles; 67000 and 100000 scans were recorded for mechanically oriented samples and bicelles, respectively. The spectra were recorded at temperatures above the gel to liquid phase transition. A 200 Hz exponential line broadening was applied before Fourier transformation.

Spectral Simulations. All spectra were simulated using Mathematica 3.0 (Wolfram Research, Champaign, IL). The CSA tensors were expressed in spherical coordinates, and spectra were calculated by integration of all possible orientations using the assumed orientational distribution as a weighting factor and folded with an inherent line broadening of 300 Hz (in addition to the Gaussian mosaicity). For the ¹⁵N backbone, the CSA tensor $\sigma_{11} = 62.5$, $\sigma_{22} = 77.3$, and $\sigma_{33} = 275$ 233.5 was taken into account,²³ where σ_{11} and σ_{33} are in the plane of the peptide bond defined by the NH vector and the C_{carboxyl}-N vector. The angle between the C_{carboxyl}-N vector and σ_{33} is 105°. For ³¹P, an axial-symmetric tensor in which the tensor elements were adjusted for the best fit was considered. The line broadening was 1600 Hz to simulate the ³¹P solid-state NMR spectra and 2 kHz for the ²H solid-state NMR spectra.

RESULTS

Solution NMR Studies in DPC Micelles. The high-resolution structure of the GLK13 peptide was determined in the presence of DPC micelles by multidimensional solution NMR spectroscopy. A combination of 2D ¹H-¹H TOCSY and 2D ¹H-¹H NOESY spectra was used to assign specific resonances and to obtain NOE connectivities. Because of the line broadening, due to the slow tumbling of peptide in micelles, the J coupling constants could not be extracted.^{24,25} Table S1 reports the resonance assignment with the exception of alanine 8, which carried a deuterated side chain. Amino acid spin systems were assigned following the sequence-specific method (NOESY-walk) reported by Wüthrich,¹⁶ and a total of 151 NOEs (81 inter-residue and 70 intra-residue) were identified. Several $d_{NN}(i, i + 1)$, $d_{\alpha N}(i, i + 1)$, and $d_{\alpha N}(i, i + 2)$ were identified along the backbone of the peptide. Panels A and B of Figure 1 show the backbone NOE pattern and the chemical shift index (CSI), respectively, for the peptide in DPC micelles. The concomitant presence of both a deviation of more than -0.1 ppm of the CSI value and the existence of $d_{\alpha N}(i, i + 3)$ and $d_{\alpha N}(i, i + 4)$ connectivities²⁶ is indicative of the presence of α -helical structures. The structures obtained from simulated annealing calculations using XPLOR-NIH confirmed this first analysis. Panels A and B of Figure 2 show the superposition of the 20 lowest-energy conformers. The reported ensemble shows an α -helical structure between Lys-5 and Lys-11 (Figure 2C), and the superposition of backbone atoms in this region

Figure 1. (A) Graphical summary of NOE constraints obtained from 1.9 mM GL13K in the presence of 189 mM DPC in 10 mM phosphate buffer (pH 5) at 298 K. (B) ^1H chemical shift index of C_αH for GL13K. Negative values are indicative of helical conformations.²⁶

Figure 2. NMR structural ensemble of GL13K in DPC micellar environments using NOE restraints represented in Figure 1. Twenty lowest-energy conformers showing the convergence for the (A) backbone and (B) side chains. (C) Representative average structure of GL13K. (D) Residue-dependent root-mean-square deviations (RMSD) for the backbone (red) and side chains (blue) comparing the 20 lowest-energy conformers. The structures were fit onto the average conformation, and residues 5–11 were used for the superposition of the conformers.

concentrations (Figure S1), and previous CD experiments 323 have indicated that the peptide only weakly interacts with 324 zwitterionic membranes.⁸ Furthermore, these experiments 325 showed that GL13K adopts β -sheet conformations upon 326 association with DOPG bilayers. To extend these previous 327 investigations, CD spectra were also recorded in the presence 328 of vesicles encompassing an intermediate amount of negatively 329 charged lipids, characteristic of bacterial membranes, namely, 330 3:1 POPE/POPG, as well as 3:1 (molar ratio) POPC/POPS 331 small unilamellar vesicles (Figure 3A,B). As in previous 332 investigations, considerable scattering hampers the evaluation 333 of spectra below 200 nm and deconvolution into different 334 secondary structures.⁸ However, the spectra closely resemble 335 those obtained in the presence of DOPG membranes where the 336 negative intensities at 217 nm were taken as an indicator of β - 337 sheet conformation.⁸ Therefore, the transition from random 338 coil in solution to β -sheet-rich conformations already occurs 339 with liposomes containing moderate concentrations of 340 negatively charged lipids. Interestingly, the transition occurs 341 in a stepwise manner once the concentration of POPE and 342 POPG exceeds 600 μM (Figure 3A). 343

The calculated net charge of GL13K is +5 at pH 7 and 0 at 344 pH 14 (pI). To further investigate the effect of peptide charge 345 on the structural preferences of GL13K, a pH titration was 346 performed by CD spectroscopy, using the pH range with the 347 highest change in net charge. A quantitative analysis of the 348 spectra at neutral pH is indicative of 10–15% α -helix, 25–30% 349 β -sheet/ β -turn, and 55% unordered (Figure 3A–C). When the 350 pH is increased to 10.6, $\sim 20\%$ of the random coil converts into 351 additional β -sheet contributions in a pH-dependent manner 352 (Figure 3C). The isosbestic point at 210 nm is indicative of a 353 two-state equilibrium. Notably, this transition was absent for 354 the randomized sequence GL13K-R1 [IGIKLLKSKLKAL- 355 NH_2 ; cf. ref 6 (Figure 3D)]. In contrast, the latter peptide 356 shows a higher helix content at high pH. 357

Solid-State NMR Studies of GL13K Associated with 358 **POPE/POPG Bilayers.** In a next step, the GL13K peptide 359 (labeled with ^{15}N at leucine 6 and with methyl-deuterated 360 alanine at position 8) was reconstituted into oriented 361 phospholipid bilayers, with a phospholipid composition 362 mimicking that of bacterial membranes, and investigated by 363 solid-state NMR spectroscopy.²⁷ The NMR parameters 364 observed in such samples are dependent on the molecular 365 alignment relative to the magnetic field direction of the NMR 366 spectrometer (B_0). When the sample normal is oriented parallel 367 to B_0 , the ^{15}N chemical shift and ^2H quadrupolar splittings can 368 be analyzed in terms of peptide topology and orientational 369 distribution.²⁷ Furthermore, the effects of the peptide on the 370 orientational order, packing, and macroscopic phases can be 371 investigated using ^{31}P solid-state NMR.²⁸ 372

Figure 4A shows the ^{31}P solid-state NMR spectrum of 3:1 373 (molar ratio) POPE/POPG bilayers in the presence of 2 mol % 374 GL13K supported by glass plates that are oriented with the 375 sample normal parallel to the magnetic field direction of the 376 spectrometer (B_0). The intensities around 30 ppm are 377 indicative of phospholipids that are oriented with their long 378 axis parallel to B_0 .²⁸ However, when compared to that of pure 379 lipid bilayers (cf. Figure 3 in ref 28), the line width of this 380 “oriented” signal intensity is quite broad (LWHH ~ 19 ppm). 381 Furthermore, a major fraction of resonances extends over the 382 full chemical shift anisotropy, where intensities close to -15 383 ppm are indicative of phospholipids in a perpendicular 384 orientation relative to the magnetic field direction (coinciding 385

311 gives a root-mean-square deviation (RMSD) of 0.29 ± 0.13 Å 312 for the backbone and a RMSD of 1.36 ± 0.40 Å for the heavy 313 atom side chains. In Figure 2D, the RMSD versus residue, blue 314 for side chains and red for the backbone, is reported.

315 **CD Spectroscopic Titrations.** The experimental con- 316 ditions used during the NMR structural analysis described 317 above are characterized by a high DPC:peptide molar ratio 318 with, on average, fewer than one peptide per micelle. The 319 helical structure thereby represents the peptides in their 320 isolated, monomeric state as they occur at a low level of 321 peptide association with, e.g., zwitterionic membranes. Indeed, 322 the helical secondary structure requires high detergent

Figure 3. CD spectral titration of 60 μM GL13K with (A) 0–3000 μM POPE/POPG (3:1 molar ratio) SUVs at 37 $^{\circ}\text{C}$ in 10 mM Tris-HCl (pH 7.4) and (B) POPC/POPS SUVs (3:1 molar ratio) at 25 $^{\circ}\text{C}$ in 5 mM Tris-HCl (pH 7.4). Spectra are corrected with control curves of SUVs only, but peptide-induced liposome aggregation causes some of the spectra to be severely affected by light scattering. Therefore, spectra at the highest lipid concentrations were truncated below 200 nm. CD spectra of (C) GL13K and (D) GL13K-R1 in buffers from pH 8.5 to 10.6. GL13K but not GL13K-R1 gradually shifts from random coil to more β -turn secondary structure with an increase in pH. GL13K-R1 but not GL13K exhibits more α -helix at high pH.

with the sample normal). Among other possibilities, the combination of 36% lipids oriented with their long axis parallel to the sample normal (18.8 ppm LWHH) and 64% randomly oriented (powder pattern) results in a related line shape. Regardless of the detailed supramolecular assembly, the spectra are indicative that the peptide has a high capacity to disrupt the membrane orientational order. In comparison, the disruption of orientational order at comparable P:L ratio is much higher for GL13K than for other cationic amphipathic antimicrobial peptides such as the helical magainins,^{28–30} phyloseptins,³¹ or the β -sheet arenicin.³² On the other hand, dermadistinctin K⁹ and protegrin³³ also show pronounced disordering effects upon being investigated in the context of supported lipid bilayers.

The ¹⁵N and ²H solid-state NMR spectra of the labeled peptide within the same sample were also investigated (Figure 4B,C). To a first approximation, the ¹⁵N spectrum represents the alignment of ¹⁵N–H vectors relative to the magnetic field direction and, thus, the main axis of helical peptides relative to the sample normal.³⁴ Whereas values of ~ 200 ppm are associated with transmembrane helical peptides (or NH vectors parallel to the sample normal), chemical shifts of <100 ppm are indicative of perpendicular orientations.³⁴ When samples are reconstituted into mechanically supported phospholipid bilayers where the sample normal is oriented parallel to the magnetic field direction, most ¹⁵N intensities appear at 85 ppm with a LWHH of ~ 47 ppm, indicating that the N–H vectors are oriented predominantly perpendicular to B_0 (Figure 4B). An angle between NH and B_0 of $\sim 100^{\circ}$ represents well the spectral maximum.

Finally, the ²H quadrupolar splitting of the ²H₃C alanine methyl groups is a function of the C_{α} – C_{β} angle relative to the magnetic field direction/sample normal and can be very sensitive to even small changes in membrane alignment.³⁵ Similar to the ³¹P solid-state NMR spectra, the ¹⁵N and ²H

spectra exhibit broad spectral line shapes that could arise from a combination of powder pattern line shapes, where the elevated shoulders at ± 35 kHz could arise from a circular orientational distribution or represent a composite of oriented contributions and powder pattern line shapes (Figure 4C). The latter contributions are expected to arise, e.g., from a twisted β -sheet.

The GL13K peptide was also investigated in the presence of phospholipid bicelles, which, at a lipid concentration of 28% (w:v), a molar ratio q ([DMPC:DMPG]/[DCPC]) of 3.5, and a temperature of 310 K, have been shown to align in the magnetic fields of NMR spectrometers.^{36,37} Interactions of the peptide with the anionic bilayer ensure that the cationic GL13K also aligns in the magnetic field. In contrast to that of the mechanically oriented sample, the bilayer normal of the bicelle is oriented perpendicular to B_0 .

Because of the fast rotation of the phospholipids around their long axis, the ³¹P NMR spectra are characterized by two components, namely, the averaged tensor elements $\sigma_{\parallel}$ and $\sigma_{\perp}$, representing the components parallel and perpendicular to the rotation axis, respectively.^{38,39} Whereas in the mechanically oriented sample, the interaction of the ³¹P chemical shift is represented by $\sigma_{\parallel}$, tilting the membrane by 90° aligns $\sigma_{\perp}$ parallel to B_0 and thereby represents the observed chemical shift. Therefore, in Figure 4D, the signal intensities at -14 and -11 ppm are indicative of oriented PC and PG components, respectively, whereas the intensity at ~ 0 ppm has been associated with the short chain DHPC lipids that freely change orientation by diffusing fast within the bicellar rim structure.^{40,41}

The ¹⁵N spectrum exhibits a broad distribution of resonances ranging from 224 to 65 ppm, indicative of NH vector orientations ranging from parallel to perpendicular relative to B_0 . Rotation around the bilayer normal, which in the bicellar case is oriented perpendicular to B_0 , results in many different

Figure 4. Solid-state NMR spectra of [^{15}N -Leu6, $^2\text{H}_3$ -Ala8]GL13K reconstituted into (A–C) mechanically oriented POPE/POPG (3:1) bilayers and (D–F) [DMPC:DMPG]/[DCPC] magnetically aligned bicelles at 310 K. The first row (A and D) shows the corresponding ^1H -decoupled ^{31}P solid-state NMR spectra of the sample phospholipids, the second row (B and E) the ^{15}N NMR spectra, and the third row (C and F) the ^2H NMR spectra obtained from the reconstituted [^{15}N -Leu6, $^2\text{H}_3$ -Ala8]GL13K peptide. The ^{31}P solid-state NMR and the mechanically oriented samples were investigated at 11.8 T, and the ^{15}N and ^2H spectra of the peptide in bicelles were recorded at 17.7 T (E and F). The dotted traces correspond to simulated spectra: (A) 36% oriented with 18.8 ppm LWHH and 64% powder with 11.8 ppm LWHH and (B) oriented with an angle between the NH bond and the membrane normal of 100° (membrane normal parallel to the magnetic field) and 47 ppm LWHH. (C) Powder pattern line shape (2/3) combined with a spectrum that results from a mean alignment of the $\text{C}_\alpha\text{--C}_\beta$ bond parallel to the membrane normal with 45° mosaicity (1/3). (E) Integral of all possible orientations with an angle between NH and the membrane normal of 100° (membrane normal perpendicular to the magnetic field) and 47 ppm LWHH. (F) Powder pattern line shape. The membrane alignment is sketched as a gray bar next to panels B and E together with the approximate alignment of the N–H bond. A sheet with the relative orientations of the NH bond and the $\text{C}_\alpha\text{--C}_\beta$ vector of the alanine side chain is shown in panel G.

the 10^4 Hz time scale of the ^{15}N chemical shift anisotropy.⁴² Finally, the ^2H solid-state spectrum of GL13K associated with the bicellar system is shown in Figure 4F. The signal:noise ratio remains modest despite 3 days of signal averaging, and therefore, the spectrum cannot be analyzed in detail. For comparison, a powder pattern line shape, making a major contribution also to Figure 4C, is shown on top of the spectrum, showing that the maximal peak intensities can be associated with such a (close to) random distribution of $\text{C}_\alpha\text{--C}_\beta$ vectors.

Finally, we used ^2H solid-state NMR spectroscopy to investigate the effect of GL13K on the fatty acyl chain packing of the bilayers. Mixed POPE/POPG membranes in which all ^1H positions of the palmitoyl chain of either POPE or POPG in the bacterial mimetic membrane were exchanged with deuterium were studied. The resulting ^2H solid-state NMR spectra encompass signals from the different CD_2 segments and one CD_3 segment, each contributing a powder pattern line shape, where the distance between the two main peaks defines the quadrupolar splitting.^{21,43} Without motions, the static splitting would amount to 125 kHz. However, in the liquid crystalline bilayers investigated here, motions and cis–trans isomerizations result in a considerable reduction, which is evident in the ^2H solid-state NMR spectra shown in panels A and C of Figure 5. Notably, the segments exhibit more motional freedom in the hydrophobic interior than the region of the carbonyls does. The order parameter profiles were extracted separately for both types of phospholipid (Figure 5B,D). In the presence of 2 mol % GL13K, a considerable decrease in the level of packing order is observed for both lipids. The decrease is $\sim 20\%$ for the POPE and 10% for POPG palmitoyl chains.

DISCUSSION

GL13K is a short cationic, amphipathic peptide that efficiently interacts with membranes. We have previously found that GL13K is effective against the Gram-negative bacterium *Escherichia coli* [minimum inhibitory concentration (MIC) of 5 $\mu\text{g}/\text{mL}$] and *Pseudomonas aeruginosa* (MIC of 8 $\mu\text{g}/\text{mL}$),⁶ whereas the peptide is not effective against the oral Gram-negative pathogen *Porphyromonas gingivalis*.⁶ Similarly, activity is limited against Gram-positive bacteria, including *Streptococcus gordonii* (MIC of 64 $\mu\text{g}/\text{mL}$) (H. Hirt and S.-U. Gorr, unpublished observations), *Staphylococcus aureus* (MIC of 41 $\mu\text{g}/\text{mL}$) (S.-U. Gorr, unpublished observations), and *Enterococcus faecalis* (MIC of >512 $\mu\text{g}/\text{mL}$) (H. Hirt and S.-U. Gorr, unpublished observations). However, the peptide kills up to 99.99% of the bacteria in existing biofilms of *P. aeruginosa*.⁷

Here we demonstrate that GL13K exhibits random coil conformations in aqueous solution, helical structures in DPC micelles, and β -sheet-rich conformations in the presence of liposomes composed of 25% negatively charged lipids, which were also observed previously upon association with DOPG liposomes.⁸ In the presence of phosphatidylcholine liposomes, the extent of membrane association of this highly charged peptide is low and the CD spectra represent mostly the peptide that remains in solution. Because light scattering interferes with optical spectroscopies, in particular when extending into the ultraviolet spectral region, only dilute liposomal suspensions have been investigated by CD spectroscopy. In contrast, during the NMR structure analysis, presented here, high DPC concentrations were used, which brings the binding equilibrium to the membrane-associated state. Therefore, the NMR

peptide orientations relative to B_0 . Indeed, taking into account the 100° NH alignment relative to the sample normal, which has been found to represent well the ^{15}N spectrum of the mechanically oriented samples (Figure 4B), also explains well the bicellar ^{15}N spectrum (Figure 4E) under the condition at which motional averaging around the bilayer normal is slow on

Figure 5. ^2H NMR spectra of $[\text{}^{15}\text{N}\text{-Leu6}, \text{}^2\text{H}_3\text{-Ala8}]$ GL13K embedded in (A) POPE/ $[\text{}^2\text{H}_{31}]$ POPE/POPG and (C) POPE/POPG/ $[\text{}^2\text{H}_{31}]$ POPG vesicles at 310 K in 10 mM Tris-HCl buffer. In each panel, the spectra in the presence of 2 mol % GL13K are shown above the spectra obtained from pure lipid bilayers. (B and D) Corresponding order parameters as a function of the carbon position on the aliphatic chain in the presence (●) and absence (○) of GL13K.

Figure 6. Model of the equilibria governing the random coil (A), the helical monomeric form (B), β -strands (C and D), and β -sheet aggregates of GL13K (E). A membrane opening and a side-on aligned β -sheet with its N–H vectors oriented parallel to the sample normal are shown in part D. Notably, parts B–E can result in membrane deformations and ultimately bilayer openings.

structural data, in combination with previously published investigations,⁸ indicate that depending on the membrane composition and other environmental parameters, GL13K adopts helical or β -sheet-rich secondary structures in the presence of membranes.

The helical structure determined in the presence of DPC micelles (detergent:peptide molar ratio of 100) extends from residue 5 to 11. The resulting three-dimensional fold results in a small hydrophobic surface of leucines 6 and 10, opposed by lysines 5, 7, and 11, which suggest an interfacial association (Figure 6). As with other cationic amphipathic peptides, the resulting hydrophobic interactions alone are weak,⁴⁴ but membrane association is enhanced by electrostatic attraction.^{8,45} Notably, in the DPC micellar system, where one micelle consists of ~ 40 detergent molecules,⁴⁶ there is on average fewer than one peptide per micelle, which probably also represents the situation in which the peptide associates with neutral membranes and the resulting low peptide:lipid ratios. Because of its weak interactions with zwitterionic membranes, the NMR structure presented here is thereby the only

biophysical data of the peptide describing the membrane-associated state at low concentrations.

In the presence of DOPG membranes, CD spectroscopy has revealed a predominantly β -sheet conformation.⁸ Clearly, the high negative charge density of this lipid bilayer increases the local peptide concentration along the membrane surface and thereby the apparent binding constant. Here we extended these investigations to intermediate concentrations of negatively charged lipids by adding 75% PE, a lipid preponderant in bacterial membranes. Also, under these conditions, CD spectra are indicative of a β -sheet conformation of GL13K (Figure 3A,B). The combination of ^{15}N and ^2H solid-state NMR data suggests a predominant alignment of the sheet along the membrane surface as depicted in Figure 4G, with considerable twist, and slow rotational diffusion around the membrane normal probably due to peptide association (Figure 4E), when at the same time the orientational order at the level of the phospholipid headgroup is severely disturbed (Figure 4A).

It is tempting to speculate that the resulting high peptide density within the membrane favors peptide–peptide interactions concomitant with β -sheet formation. The data do not

reveal the oligomerization state of GL13K in the membrane. However, it should be mentioned that the appearance of ^{15}N and ^2H spectra varies with the solvent used during the sample preparation, suggesting that the equilibria between mono- and oligomeric states are affected. On a related note, increasing the pH in the absence of membranes results in the formation of β -sheet structures (Figure 3C), whereas the randomized GL13-R1 sequence exhibits additional α -helical features (Figure 3D). Such data indicate that a propensity for β -sheet is inherent in the detailed amino acid arrangement of GL13K, whereas the amino acid composition per se is in agreement with helical secondary structures.

In analogy with previous investigations,^{32,47} the ensemble of data suggests that the GL13K–membrane interactions are governed by a number of equilibria encompassing random coil, α -helical, and β -turn conformations as well as β -sheet aggregates (Figure 6). Catestatin has similarly been found to encompass a seven-residue α -helical conformation in micellar solutions with the remainder of the 21-residue peptide unstructured.⁴⁸ In contrast, the shortened cateslytin analogue exhibits CD spectra characteristic of β -turn sheet conformations in the presence of negatively charged liposomes.⁴⁹ Notably, the carboxy-terminal residue of cateslytin coincides with the helical boundary of catestatin, suggesting that the missing residues stabilize the helical domain of catestatin. As a result of the missing intramolecular H-bonds typical for helical structures, an intermolecular H-bonding network forms in the case of cateslytin. The cateslytin sequence and the GL13K peptides share a similar size (15 and 13 amino acid residues, respectively), a high density of positive charges (5+), and an α -helix– β -sheet transition/equilibrium in membrane environments (Figure 6). The fusion peptide of HIV has also been found to exhibit an equilibrium between α -helical and β -turn secondary structures that is dependent on the lipid composition.⁵⁰

In its helical conformation, the peptide probably interacts with the membrane interface through hydrophobic (three or four leucines) and electrostatic interactions (four lysines and the amino terminus), which results in a wide range of topologies (Figure 4B,C). At the same time, the formation of aggregates is favored by high peptide densities at the membrane and the screening of excess positive charges on the peptide by anionic PG headgroups. Thereby, the high surface concentration in the presence of POPG competes efficiently with helical membrane partitioning and results in the formation of β -sheets. On a related note, the β -sheet secondary structures observed at high pH are probably a result of weakened repulsive electrostatic contributions (Figure 3C).

Importantly, many cationic linear AMPs have been suggested to kill bacteria by forming openings in the cytoplasmic membranes that lead to the collapse of the electrochemical gradient and the release of intracellular contents. They do so by inserting their amphipathic structures into the membrane surface rather than by fully spanning the lipid bilayers.^{45,51} Potential mechanisms that have been proposed to explain the molecular structure of such membrane openings include, first, the formation of a barrel stave arrangement (cf. refs 32 and 47).

Second, the 15-residue cationic peptide cateslytin has been suggested to form β -sheets oriented along the membrane surface. At peptide:lipid ratios of 1:15, these were shown to selectively interact with anionic lipids, to cause an increased level of order of the fatty acyl chains of negatively charged lipids and lateral phase separation of 1:1 DMPC/DMPS mem-

branes.⁴⁹ The packing defects along the resulting phase boundaries are thought to be susceptible to membrane passage. Notably, here we found a strong membrane disordering effect, which is stronger on the zwitterionic PE than on the PG component of the mixed membranes (Figure 5).

Third, the partitioning of amphipathic peptides into the membrane interface has previously been shown to cause considerable curvature strain and to disrupt membrane packing.⁴⁵ In the case of GL13K, this effect is quite pronounced both when the global order of the oriented membranes is monitored by ^{31}P solid-state NMR spectroscopy (Figure 3A) and when the fatty acyl chain order is investigated (Figure 5). It is thought that this can be due to molecules that partition in the membrane interface, which thereby increase membrane curvature strain (Figure 6). Interestingly, the membrane disordering effect is more pronounced for POPE than for POPG in mixed membranes, which is surprising considering that other cationic peptides have been shown to preferentially associate with the negatively charged lipid in PC/PS/cholesterol membranes.⁵²

Our results do not directly address the activity of GL13K in the more complex environment of bacterial biofilms that are organized in an extracellular matrix. However, the peptide–peptide interactions concomitant with β -sheet formation and the resulting membrane disruption may also affect bacterial interactions and disrupt their organization in biofilms. Indeed, when biofilms of *P. aeruginosa* are treated with GL13K, the bacteria are killed in place and the biofilm appears to be destabilized and is dispersed or shed from the surface, as evidenced by scanning electron microscopy.⁷

Selectivity for bacterial versus healthy eukaryotic cells arises from the high negative surface charge and the high transmembrane electrical potential that lead to an increased concentration of the peptides along the prokaryotic surface.^{53,54}

Therefore, the α -helical structure at the low peptide:lipid ratios that occur in the presence of zwitterionic membranes versus the β -sheet conformations observed upon exposure to anionic lipids also reflects the selectivity of GL13K for bacterial cells and its lack of toxicity against eukaryotic cells. Furthermore, cholesterol protects the membranes of eukaryotic organisms. In general, the mode of interaction depends on peptide structure (distribution of hydrophilic and hydrophobic amino acids), peptide concentration, and the physicochemical properties of the membrane (concept reviewed in ref 45), suggesting that a delicate balance of interactions needs to be considered.

In conclusion, whereas the highly charged GL13K peptide is strongly attracted to negatively charged membranes, hydrophobic association is rather weak. At low peptide:detergent/lipid ratios, the peptide adopts a helical conformation upon interacting with membranes, which converts to β -sheet-rich supramolecular arrangements with highly membrane disruptive properties. The high charge and small hydrophobic phase of this short peptide antibiotic thereby result in high conformational plasticity.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00526.

685 NMR resonance assignments, statistics for the structure
686 calculations, and a CD spectroscopic titration of DPC
687 into a GL13K solution (PDF)

3-phosphoserine; rh, relative humidity; SDS, sodium dodecyl
sulphate; TFA, trifluoroacetic acid; TOCSY, total correlation
spectroscopy.

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700 work.

701 Funding

702 This research was supported by Grant 1R01DE017989 from
703 the National Institute for Dental and Craniofacial Research (S.-
704 U.G.), Grant GM 64742 from the National Institute for
705 General Medical Sciences (G.V.), and a fellowship supported
706 by Grant R90DE023058 from the National Institute of Dental
707 and Craniofacial Research (X.C.). Research funds were
708 provided by the University of Minnesota School of Dentistry
709 (S.-U.G.) and The Office of the Vice-president for Research at
710 the University of Minnesota, Project 55466 of the Grant-in-Aid
711 of Research, Artistry and Scholarship Program (C. Aparicio).
712 B.B. was supported by a sabbatical stay at the University of
713 Minnesota School of Dentistry that was generously supported
714 by the School's Lasby Visiting Professor Fellowship and the
715 University of Strasbourg. B.B. received additional support from
716 the Agence Nationale de la Recherche (Projects TRANSPEP
717 07-PCV-0018, membraneDNP 12-BSV5-0012, and MemPep-
718 Syn 14-CE34-0001-01 and the LabEx Chemistry of Complex
719 Systems 10-LABX-0026_CSC), the RTRA International
720 Center of Frontier Research in Chemistry, the French
721 Foundation for Medical Research (FRM), the University of
722 Strasbourg, the CNRS, and the Région Alsace.

723 Notes

724 The authors declare no competing financial interest.

725 ■ ACKNOWLEDGMENTS

726 We are grateful to Delphine Hatey for the preparation of
727 peptides.

728 ■ ABBREVIATIONS

729 CD, circular dichroism; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-
730 phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phospho-
731 choline; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-(1'-*rac*-glycerol)
732 sodium salt; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine;
733 DOPG, 1,2-dioleoyl-*sn*-glycero-3-(1'-*rac*-glycerol) sodium salt;
734 DPC, dodecylphosphocholine; HPLC, high-performance liquid
735 chromatography; LWHH, line width at half-height; MAS,
736 magic angle spinning; NMR, nuclear magnetic resonance;
737 NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser
738 effect spectroscopy; MALDI, matrix-assisted laser desorption
739 ionization; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-
740 choline; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoetha-
741 nolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(1'-*rac*-
742 glycerol) sodium salt; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-

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