Synergistic Transmembrane Alignment of the Antimicrobial Heterodimer PGLa/Magainin*

Received for publication, May 18, 2006, and in revised form, July 20, 2006. Published, JBC Papers in Press, July 28, 2006, DOI 10.1074/jbc.M604759200

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The antimicrobial activity of amphiphilic α-helical peptides is usually attributed to the formation of pores in bacterial membranes, but direct structural information about such a membrane-bound state is sparse. Solid state 2H-NMR has previously shown that the antimicrobial peptide PGLa undergoes a concentration-dependent realignment from a surface-bound S-state to a tilted T-state. The corresponding change in helix tilt angle from 98 to 125° was interpreted as the formation of PGLa/magainin heterodimers residing on the bilayer surface. Under no conditions so far, has an upright membrane-inserted I-state been observed in which a transmembrane helix alignment would be expected. Here, we have demonstrated that PGLa is able to assume such an I-state in a 1:1 mixture with magainin 2 at a peptide-to-lipid ratio as low as 1:100 in dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol model membranes. This 2H-NMR analysis is based on seven orientational constraints from Ala-3,3,3-d3 substituted in a non-perturbing manner for four native Ala residues as well as two Ile and one Gly. The observed helix tilt of 158° is rationalized by the formation of heterodimers. This structurally synergistic effect between the two related peptides from the skin of Xenopus laevis correlates very well with their known functional synergistic mode of action. To our knowledge, this example of PGLa is the first case where an α-helical antimicrobial peptide is directly shown to assume a transmembrane state that is compatible with the postulated toroidal wormhole pore structure.

Membrane-active antimicrobial peptides with typically 10–50 amino acids are produced by many organisms as part of the immune defense against bacteria and other microorganisms (1, 2). These peptides kill bacteria presumably by disrupting their cell membranes. To understand their detailed mode of action at a molecular level, it is important to examine their structures in association with lipid membranes, for which solid-state NMR is particularly well suited (3).

The peptides PGLa and magainin 2 (MAG)2 are found in the skin of the frog Xenopus laevis and represent some of the first antimicrobial peptides discovered (4–7). MAG, in particular, has been studied extensively (see Refs. 8 and 9 for reviews). Both peptides occur in the same biological tissue, and several studies have demonstrated synergistic effects in their antimicrobial action. For example, the membrane potential decreases when either PGLa or MAG is added to bacteria, and a greatly enhanced effect is observed for mixtures (10). Likewise, liposome leakage induced by MAG was found to increase 25–40-fold in the presence of a small amount of PGLa (11). Synergism has also been demonstrated in studies of respiration in spermatozoa (12) and membrane permeability (13) for 1:1 mixtures of PGLa and MAG. A functional study on anti-tumor effects, membrane leakage, and glucose permeability of several mixtures with different ratios shows that the 1:1 mixture is the most potent, suggesting heterodimer formation (14). Structure analysis by circular dichroism and fluorescence spectroscopy suggests dimerization via specific interactions between the two peptides, as amino acid mutations change their mutual affinity (15). A cross-linking study shows that heterodimers are formed in a parallel fashion, and the cross-linked peptides are more efficient in inducing vesicle leakage (16).

In the present analysis, we used solid-state NMR to determine the molecular alignment of PGLa in lipid bilayers in the presence of MAG. In determining whether PGLa has a preference for forming heterodimers with MAG rather than homodimers, we expected to see structural differences compared with its usual behavior, which we had characterized in detail previously (17–21). The amino acid sequences (see Table 1) suggest that both peptides form amphiphilic α-helices, with charged and polar side chains on one face and hydrophobic residues on the other (see helical wheels in Fig. 1). For PGLa, the α-helical conformation is confirmed by circular dichroism in the presence of lipid vesicles (15, 20, 22) and by 1H-NMR in detergent micelles (23). MAG is also known to form an α-helix when bound to membranes (24).

Solid-state NMR is a powerful tool in resolving the structure and alignment of membrane-active peptides in lipid bilayers (3, 25). For simple α-helical peptides, it is straightforward to collect a number of orientational constraints from which the molecular conformation can be verified and its membrane alignment and dynamic behavior deduced (26). For each NMR constraint, a selective isotope label has to be placed into a suitable position on the peptide. Non-perturbing 15N isotopes are conveniently incorporated into the backbone and 2H-labels into the side chains. Alanine-3,3,3-d3 (Ala-3,3,3-d3) is highly suitable, because the deuterated methyl group is attached directly to the
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backbone and reflects the orientation of the entire peptide segment (27, 28).

Previous 15N-NMR studies of PGLa have shown that the helix axis is aligned roughly parallel to the membrane surface (20, 23). Analogous studies on MAG have also revealed a flat S-state in various lipid systems up to a peptide-to-lipid molar ratio (P/L) of 1:25 (29–32). For our more recent 2H-NMR studies, we have placed selective Ala-d3 labels into PGLa and measured its alignment more accurately in dimyrystophosphatidylcholine (DMPC) and in mixed DMPC/DMPG (dimyrystophosphatidylglycerol) bilayers. A slight but significant concentration-dependent realignment of the helix axis in the membrane has been observed (17, 18). The same realignment has also been characterized by 19F-NMR studies of 19F-labeled PGLa (19–21). At low peptide concentration, the helix axis was found to lie almost flat on the membrane surface in the so-called S-state at an angle of 98° with respect to the membrane normal. At high peptide concentration, an obliquely tilted T-state was discovered and characterized for the first time. The helices were tilted by ~125° in this putative antiparallel homodimer. We did not, however, observe any upright transmembrane orientation that would be expected for an inserted I-state (where the tilt angle would be near 0 or 180°), which is proposed in the Matsuzaki-Shai-Huang model of antimicrobial activity (8, 33, 34). In the I-state, the peptides are supposed to form transmembrane pores, which induce cell leakage and thereby kill bacteria. Such pores have been observed by neutron diffraction for MAG in the DMPC/DMPG (3:1) system (35), where the peptide was proposed to be in the I-state.

In the present study, Ala-d3-labeled PGLa was reconstituted with MAG at a 1:1 molar ratio into DMPC/DMPG model membranes. A solid-state 2H-NMR structure analysis was performed to determine the orientation of PGLa in the presence of MAG to examine the possible formation and structural behavior of synergistically active heterodimers.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—PGLa was labeled at eight different positions, one at a time, replacing Ala, Gly, or Ile with Ala-d3 (see Table 1). MAG was prepared without isotope labels. All peptides were chemically synthesized and purified as described previously (17, 18). The identity of the products was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and analytical high pressure liquid chromatography showed them to be at least 95% pure.

**Sample Preparation**—DMPC and DMPG were purchased from Avanti Polar Lipids (Alabaster, AL). Macroscopically oriented membranes on glass plates were prepared as previously described (17, 18). Appropriate amounts of peptides and lipids were co-dissolved in ~400 μl of methanol/CHCl3 (1:1 v/v) and spread onto 20 thin glass plates of dimensions 18 × 7.5 × 0.08 mm3 (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). The plates were dried in air for 1 h followed by drying under vacuum overnight. They were then stacked and hydrated using deuterium-depleted water at 96% relative humidity at 48 °C for 24–48 h.

**NMR Spectroscopy**—All solid-state NMR measurements were carried out on Bruker Avance 500- or 600-MHz spectrometers at 308 K. 31P- and 2H-NMR experiments were performed as previously described (17, 18). The structure analysis from the 2H-NMR data was performed as previously described for PGLa (17, 18, 20, 21, 28). This method is described in depth in several recent reviews (3, 26, 36).

**RESULTS**

**Choice of Peptide-Lipid System**—For PGLa in DMPC bilayers, we have found previously that the peptide binds in a monomeric S-state at P/L ≤ 1:200, whereas at P/L ≥ 1:50, a homodimERIC T-state is characterized in oriented NMR samples (17, 21). In a negatively charged lipid mixture of DMPC/DMPG (3:1), the same realignment of PGLa was observed as in zwitterionic DMPC, although the T-state was reached at a somewhat lower peptide concentration. The exact threshold concentration depends on the affinity of the cationic peptide to the lipids and on the presence of excess water in the sample (18). Therefore, in the present study, a PGLa/MAG/DMPC/DMPG (1:1:75:25) system was used to prepare oriented NMR samples (containing no excess water). This composition corresponds to a total P/L of 1:50, as the individual peptides contribute with a P/L of 1:100 each. PGLa alone has been shown to exist in the T-state both at P/L = 1:50 and 1:100 in both DMPC and DMPG (3:1), which implies that PGLa forms stable homodimers under all these conditions (18).

**31P-NMR Quality Check**—The oriented membrane samples were examined by 31P-NMR and found to form a well oriented lamellar phase (Fig. 2A). A simulation of the spectrum was performed with the program DMFit (37), and it was found that ~80% of the signal came from oriented parts, with a ratio of 3:1 from DMPC and DMPG, respectively, and 20% of the signal coming from unoriented parts of the sample. Thus, no enrichment of either lipid was found in the unoriented part. Neither non-lamellar or narrowed signals were observed.

**2H-NMR Analysis**—The 2H-NMR spectra of PGLa/MAG/ DMPC/DMPG (1:1:75:25) with PGLa labeled at eight different positions with Ala-d3 are shown in Fig. 2B. The respective 2H quadrupole splittings are listed in Table 1. Because the splittings for any one label are different in the PGLa and PGLa/MAG samples, this clearly indicates a different alignment of PGLa in the lipid bilayer when MAG is present, suggesting that heterodimers are formed.
To calculate the alignment of PGLa in the heterodimer from the NMR data of Fig. 2B, we note that a helical peptide in the membrane can be generally described by three parameters: the tilt angle $\tau$ of the helix axis with respect to the bilayer normal, the azimuthal rotation angle $\rho$ around the helix axis, and the molecular order parameter $S_{\text{mol}}$ (3, 20, 21, 26–28, 36, 38). Given that the effective (time-averaged) quadrupole tensor is collinear with the C–CD$_3$ axis in the molecular frame of PGLa, at least three orientational constraints are required to calculate the three parameters $\tau$, $\rho$, and $S_{\text{mol}}$. In practice, more than three are needed, as the sign of a quadrupole splitting is not accessible, which leads to multiple solutions (39). A previous study indicates that at least four labeled positions are required to obtain a reliable structure (28). To perform this kind of analysis, the secondary structure of the peptide in the lipid bilayer has to be known. PGLa has been previously shown by circular dichroism and $^1$H-NMR to form an $\alpha$-helix in the region between residues 6–21 (15, 20, 22, 23), where our Ala-$d_3$ labels were placed. We therefore used an ideal poly(alanine) $\alpha$-helix as a model structure for PGLa, and this model was fitted to the experimental $^2$H-NMR data of the peptide. In a grid search for the best-fit structure, the theoretical quadrupole splittings were systematically calculated for all combinations of $\tau$, $\rho$, and $S_{\text{mol}}$ (17, 18, 21). The parameters $\tau$ and $\rho$ were changed in steps of 1° and $S_{\text{mol}}$ in steps of 0.01 to find the lowest root mean square deviation with regard to the experimental data. The results were then displayed in a quadrupolar wave plot to assess the deviations of the individual data points and in a two-dimensional $\tau$-$\rho$ map to check whether there were any further local minima present.

**PGLa Alignment in the Presence of MAG**—A substitution of Ala by Ala-$d_3$ is entirely non-perturbing and does not affect the molecular behavior of wild-type PGLa. When any other amino acid (in this case a single Gly or Ile) is replaced by Ala-$d_3$, such a mutation might change the properties of the peptide. Therefore, the $^2$H-NMR data analysis was first performed by taking into account only those four orientational constraints from the non-perturbed native Ala positions in the sequence, i.e. using the quadrupole splittings of Ala-6, -8, -10, and -14. That way, we found a helix tilt angle of $\tau = 158^\circ$ for PGLa in the 1:1 mixture with MAG, corresponding to an almost upright transmembrane alignment of the peptide. The best-fit parameters are listed in Table 2, and a quadrupolar wave plot is shown in Fig. 3A, (solid line). In this representation, the hypothetical quadrupole splittings are calculated for each position around the helical wheel and displayed on a curve from 0 to 360°. For comparison, we recall that the tilt angle of PGLa alone in an analogous DMPC/DMPG system had been previously determined to be $\sim 125^\circ$ with respect to the membrane normal (18) (Table 2).

To verify and strengthen this result, additional Ala-$d_3$ labels were included in the structure analysis, replacing either Gly-7, Ile-9, or Ile-13. The combined fit over seven data points, including these three labels, gave almost the same best-fit values in $\tau$, $\rho$, and $S_{\text{mol}}$ (Table 2; Fig. 3A, dashed curve). None of the seven imposed (dashed line), which corresponds to 20% unoriented signal and 80% oriented with a 3:1 ratio between the DMPC- and DMPG-oriented lines. ppm, parts/million.

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**FIGURE 2.** $^{31}$P-NMR spectrum (A) and $^2$H-NMR spectra (B) of PGLa/MAG/DMPC/DMPG in a 1:1:75:25 ratio in which PGLa is labeled with Ala-$d_3$ in eight different positions numbered according to the peptide sequence (numbers to the left of the spectra). The NMR samples were prepared as macroscopically oriented membranes between glass plates and measured with the bilayer normal parallel to the external magnetic field at 308 K. In A, one representative $^{31}$P-NMR spectrum (solid line) is shown for PGLa7, as the other samples gave similar line shapes. A simulated line shape is superimposed (dashed line), which corresponds to 20% unoriented signal and 80% oriented with a 3:1 ratio between the DMPC- and DMPG-oriented lines. ppm, parts/million.
TABLE 1
Amino acid sequences of the peptides used and ²H-NMR quadrupole splittings (in kHz) of the peptide/lipid samples

| Peptide     | Labeled position | Sequence                       | Quadrupolar splitting |
|-------------|------------------|--------------------------------|-----------------------|
| MAG wild type | None             | GMASKAGAIAGKIAKVALKAL-NH₂     |                       |
| PGLa wild type | None             | GMASKAGAIAGKIAKVALKAL-NH₂     |                       |
| PGLa6       | Ala-6            | GMASK-A-Ala-d₃ - GAIAKGKIAKVALKAL-NH₂ | 17.5                 |
| PGLa7       | Gly-7            | GMASKA-A-Ala-d₃ - AIAKGKIAKVALKAL-NH₂ | 3.0⁴                  |
| PGLa8       | Ala-8            | GMASKA - Al₃ - GAIAKGKIAKVALKAL-NH₂ | 39.2                 |
| PGLa9       | Ile-9            | GMASKAIG - Al₃ - AIAKGKIAKVALKAL-NH₂ | 25.5                 |
| PGLa10      | Ala-10           | GMASKAGAIG-Ala-d₃ - KIAKGKIAKVALKAL-NH₂ | 26.5                 |
| PGLa11      | Gly-11           | GMASKAIGAI - Ala-d₃ - KIAKGKIAKVALKAL-NH₂ | 30.5                 |
| PGLa12      | Ile-13           | GMASKAIGAI - Ala-d₃ - AIAKGKIAKVALKAL-NH₂ | 1.0⁴                 |
| PGLa13      | Ala-14           | GMASKAIGAI - Ala-d₃ - KIAKGKIAKVALKAL-NH₂ | 32.7                 |
| PGLa14      | Ala-14           | GMASKAIGAI - Ala-d₃ - KIAKGKIAKVALKAL-NH₂ | 18.6                 |

¢ PGLa/DMPC/DMPC (2:75:25) (data from Ref. 18).
° PGLa/MAG/DMPC (1:1:75:25); spectra in Fig. 2.
* PGLa/DMPC 1:50 (data from Ref. 18).
 Plan position Gly-11 was replaced by Ala-d₃, as this splitting was inconsistent with the remaining data (Fig. 3A). When this data point was included in the mathematical data analysis, virtually the same tilt angle was calculated, but with a high S_-mol and a very high root mean square deviation (Table 2).

![Graph](image)

The quality and uniqueness of the fitted solution could be assessed using two-dimensional $\tau$-$\rho$ error plots. Fig. 3B shows the error plot for the fit using only the four native Ala-d₃ labels. The root mean square deviation (in kHz) is illustrated by a gradient gray scale for all combinations of $\tau$ and $\rho$. There exist several minima with a root mean square deviation <2.0 kHz, as the signs of the quadrupole splittings are not known, but the solution with a tilt angle of $\tau = 158^\circ$ is clearly the best fit ($\rho = 93^\circ$, $S_-mol = 0.84$). The corresponding quadrupolar wave in Fig. 3A, solid line, represents the non-perturbed peptide structure. When the data from Gly-7, Ile-9, and Ile-13 are included (Fig. 3A, dashed line), the error plot in Fig. 3C then shows only one distinct minimum, proving that this is the unique solution.
DISCUSSION

In this solid-state $^2$H-NMR study, we have labeled PGLa selectively with Ala-$d_3$ and determined the orientation of this antimicrobial peptide in a lipid bilayer in the presence of MAG. Besides placing the labels into native Ala positions, in some cases, Ala-$d_3$ was also used to replace an Ile or Gly residue. Antimicrobial tests performed on these mutated peptides showed that they all have the same activity as wild-type PGLa within a factor of two, and all of the mutants also showed synergistic effects when mixed with MAG.3 To be sure to avoid any potential conformational artifacts induced by the mutations, in the first round of the data analysis, we used only the non-perturbed peptides, and we then refined the analysis by including the mutants.

In the following discussion, we will first compare the observed alignment of PGLa with and without MAG. Thereafter, we will discuss the possibility that toroidal wormhole pores are formed in the membrane. Finally, we will examine the possible role of specific interactions between PGLa and MAG.

Previous studies of PGLa in different lipid systems (DMPC and DMPC/DMPG) over a wide range of concentrations from P/L = 1:200 to P/L = 1:20 always reveal an S-state or a T-state alignment or a fast averaging between the two (17, 18, 20, 21). The S-state is attributed to monomeric peptides lying virtually flat on the membrane surface and rotating fast around the membrane normal with the lysine side chains pointing toward the water phase (17, 18, 20, 21). The T-state, observed at a higher peptide concentration, exhibits a tilt angle of ~125°, and the charged amino acids also point toward the aqueous phase (17, 18, 21). This alignment corresponds to a helix inclination of ~35° relative to the membrane plane, with the C terminus penetrating more deeply than the N terminus. The T-state is proposed to represent antiparallel homodimers engaged in fast rotation around the membrane normal. It corresponds to a distinct and stable state, presumably because of a specific packing of the two PGLa helices. Under certain conditions, the T-state has been observed to exist in fast exchange with the S-state (18).

In the present study, Ala-$d_3$-labeled PGLa was mixed with unlabeled MAG at a 1:1 molar ratio, and the splittings from $^2$H-NMR were analyzed. In the presence of MAG, we discovered a distinctly different helix tilt angle for PGLa of ~158°. This nearly upright alignment of PGLa suggests a membrane-inserted I-state, which has been postulated to be responsible for antimicrobial action (8, 33, 34). A formation of membrane-inserted complexes would explain the observation that PGLa/MAG mixtures are less sensitive to proteases than either peptide alone (14). The functional synergism with a maximum effect at a 1:1 molar ratio observed in previous studies (12–15) also supports our interpretation that PGLa/MAG heterodimers are present in the NMR sample. The corresponding tilt angle of 158° would give PGLa an effective length across the membrane of ~21 × 1.5 × cos(158°) Å = 29.2 Å, assuming that the 21-residue peptide forms an α-helix with a 1.5-Å step distance/residue. This length is enough to span the DMPC/DMPG bilayer, assuming a hydrophobic thickness of ~26 Å, close to that of pure DMPC (40).

In a transmembrane alignment, the amphipathic PGLa/MAG dimers are expected to assemble further to avoid any contact of the charged amino acids with the hydrophobic bilayer interior. Because the $^{31}$P-NMR spectra showed that all of the lipids were in a lamellar phase, the formation of small or non-lamellar aggregates can be excluded. Therefore, the heterodimers presumably assemble into larger transmembrane pores in which the charged residues line the water-filled channel, as schematically illustrated in Fig. 4. For PGLa/MAG, a higher order parameter ($S_{mol} = 0.8$) was found than for PGLa alone ($S_{mol} = 0.7$). This reduced wobble of PGLa/MAG also supports the formation of larger assemblies. We also observed that the usual long-axial rotation of PGLa around the bilayer normal ceases in the presence of MAG. This was deduced from measurements of the oriented NMR sample at a 90° inclination with respect to the static magnetic field. Transmembrane pores have been previously observed for MAG using neutron diffraction (41), and a toroidal wormhole model has been proposed (8). The pore was postulated to be lined by both negatively charged lipid headgroups as well as cationic peptides, as opposed to the barrel-stave model in which only peptides line the pore. Such wormholes have also been proposed for PGLa alone, but so far no direct evidence has been presented. $^{31}$P-NMR line shapes can provide some information about the lipid behavior and morphology. Our spectra are similar to those reported in the presence of the antimicrobial peptide MSI-78, for which wormhole formation has been proposed (42).

Having attributed the synergistic action of PGLa/MAG to the formation of stable transmembrane pores, we still need to explain the lower but yet considerable activity of PGLa alone, which was always found to remain surface-bound in our NMR analysis. One possibility is that the homodimers of PGLa with a shallow tilt angle preferentially float on the membrane surface but occasionally assemble further to form a short-lived pore in the I-state. When MAG is added, heterodimers form instead, for which the corresponding pores are more stable and sufficiently long-lived to be observed by NMR (see Fig. 4). A likely factor contributing to their stability could be the reduced electrostatic repulsion of MAG (+3 to +4) compared with PGLa (+5). Indeed, Matsuzaki et al. (15) have demonstrated by fluo-

3 P. Tremouilhac, D. Tiltak, E. Strandberg, P. Wadhwani, and A. S. Ulrich, unpublished results.
rescence quenching that the lifetime of pores follows the order of PGLa > PGLa/MAG > PGLa (15).

Concerning the specific interaction between PGLa and MAG, we speculate that the heterodimers are stabilized by salt bridges between the negatively charged Glu-19 of MAG and one of the lysines of PGLa, most likely Lys-19 or Lys-5, for the case of parallel and antiparallel dimers, respectively. A cross-linking study, where Cys residues had been added to the termini of the peptides in a systematic way, shows that heterodimers form in lipid membranes in a parallel fashion (16), which suggests the formation of parallel dimers.

We also note that the $^1H$ quadrupole splitting of Ala-δ$_3$ substituted for position Gly-11 in PGLa did not fit with the other seven data points. This deviation may be attributed to a specific interaction between PGLa and MAG at this position, which gets perturbed in the Gly→Ala mutant. Gly-Gly interactions have indeed been shown to be important for dimerization in other membrane-associated peptides and proteins (43).

Acknowledgments—We are grateful to Olaf Zwernemann, Silvia Gehring and Stephanie Maurer at the Institute for Biological Interfaces at Forschungszentrum Karlsruhe for their help with synthesis and purification of PGLa and MAG.

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