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Synergistic Insertion of Antimicrobial Magainin-Family Peptides in Membranes Depends on the Lipid Spontaneous Curvature

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ABSTRACT PGLa and magainin 2 (MAG2) are amphiphilic antimicrobial peptides from frog skin with known synergistic activity. The orientation of the two helices in membranes was studied using solid-state ^{15}N-NMR, for each peptide alone and for a 1:1 mixture of the peptides, in a range of different lipid systems. Two types of orientational behavior emerged. 1), In lipids with negative spontaneous curvature, both peptides remain flat on the membrane surface, when assessed both alone and in a 1:1 mixture. 2), In lipids with positive spontaneous curvature, PGLa alone assumes a tilted orientation but inserts into the bilayer in a transmembrane alignment in the presence of MAG2, whereas MAG2 stays on the surface or gets only slightly tilted, when observed both alone and in the presence of PGLa. The behavior of PGLa alone is identical to that of another antimicrobial peptide, MSI-103, in the same lipid systems, indicating that the curvature-dependent helix orientation is a general feature of membrane-bound peptides and also influences their synergistic intermolecular interactions.

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The two antimicrobial peptides PGLa and magainin 2 (MAG2) from the African frog *Xenopus laevis*, which are active against Gram-positive and Gram-negative bacteria, show intriguing synergistic effects that are not yet well understood (1). Structural insights into this synergy may help in the development of a new antibiotic-combination therapy. Both peptides are known to form α-helices when bound to lipid bilayers (2–4). The orientation of such α-helices in a membrane can be readily determined from the ^{15}N-NMR chemical shift in oriented lipid bilayers that are aligned with the sample normal parallel to the external magnetic field (5). If the ^{15}N chemical shift is ~90 ppm, the peptide lies flat on the membrane surface (in the so-called S-state). On the other hand, when the ^{15}N chemical shift is ~200 ppm, the peptide is fully inserted (the I-state) in a transmembrane alignment. For intermediate orientations, where the peptide is tilted (the T-state) with an angle of typically 30°–60° relative to the membrane normal, intermediate chemical shifts are expected, but the exact tilt angle cannot be determined from a single label in such cases (6). Using several selectively ^{2}H- or ^{19}F-labeled peptide analogs, more exact orientations can be obtained, since both the tilt and the azimuthal angles can be measured with high accuracy, and valuable information about dynamics also can be deduced (3,7–10).

The orientation of PGLa and MAG2 in membranes has been extensively studied with solid-state NMR, and some clues about the synergistic mechanism have been observed. Notably, in DMPC/DMPG membranes, it has been shown that the peptides on their own are in the S-state or T-state, but when the peptides are mixed in a 1:1 molar ratio, PGLa changes to the I-state, whereas MAG2 stays on the membrane surface (1,11,12). Thus, in the mixed system, transmembrane pores, which would not be spontaneously formed by each peptide on its own, appear to be stable, which could be the basis for synergy. On the other hand, it was also reported that in POPC/POPG there is no change in orientation when PGLa and MAG2 are mixed, as both peptides stay always in the S-state (12). This observation was attributed to the greater hydrophobic thickness of the POPC/POPG bilayer, compared to the DMPC/DMPG bilayer, suggesting that the PGLa helix is so short that it can only insert into thin DMPC/DMPG membranes. However, we have recently shown for MSI-103, a designer-made antimicrobial peptide based on the PGLa sequence, that the orientation determined by ^{2}H-NMR depends not on the bilayer thickness but rather on the intrinsic spontaneous curvature of the lipids (13). Accordingly, an insertion of PGLa and MAG2 into POPC/POPG should be prevented by the pronounced negative spontaneous curvature induced by the unsaturated acyl chains. However, a simple comparison of only two lipid systems does not yield an answer as to which of these two hypotheses is correct. Therefore, we have now collected data over a wide range of lipid systems, with systematic variations of acyl chain lengths (to address

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bilayer thickness) as well as chain saturations (to address lipid curvature) (see Table S1 in the Supporting Material). In this way, we found unambiguously that lipid curvature is also the decisive factor in the insertion of PGLa/MAG2.

Here, ^{15}N-NMR spectra of singly labeled peptides were recorded, and for each liquid-crystalline lipid system we prepared four oriented samples: ^{15}N-MAG2 alone, ^{15}N-MAG2 with PGLa, ^{15}N-PGLa with MAG2, and ^{15}N-PGLa alone. The total peptide/lipid molar ratio (P/L) was 1:50. ^{15}N-NMR spectra are shown in Fig. 1, and the chemical shifts are listed in Fig. 2. The quality of each oriented sample was checked with ^{31}P-NMR (see Fig. S1). Our previous detailed ^{2}H- and ^{19}F-NMR analysis of PGLa in DMPC and DMPC/DMPG showed that the helix realigns depending on the peptide concentration. Namely, at low concentration, PGLa is in an S-state with a tilt angle of ~98° (3), but above a threshold concentration around P/L = 1:100, it flips into a tilted T-state with a tilt angle of ~125° (9,14). In the presence of MAG2, it was found that PGLa inserts almost upright in an I-state with a tilt angle of ~158° (11). The present ^{15}N-NMR study confirms that in DMPC/DMPG (3:1), PGLa is in the I-state when mixed with MAG2, as indicated by the ^{15}N chemical shift of 205 ppm. PGLa alone at P/L = 1:50 has a chemical shift of 116 ppm, which corresponds to a tilted orientation, as expected. MAG2 alone is found to be in the S-state (91 ppm), but when it is mixed with PGLa its signal moves to 105 ppm, indicating a small change in the alignment. MAG2 is, however, clearly not inserted like PGLa.

In thin DLPC bilayers (12 carbon atoms in the chains) we see a behavior similar to that in DMPC (14 carbons), even though the exact chemical shifts are slightly different. PGLa alone is in the T-state, but in combination with MAG2, it flips into the I-state. MAG2, on the other hand, stays in the S-state with and without PGLa. In DPPC bilayers (16 carbons) also, the behavior is similar. PGLa alone

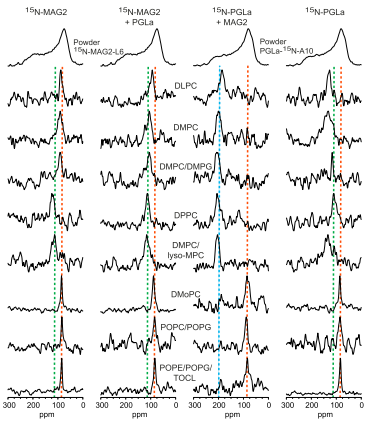

FIGURE 1 ^{15}N-NMR spectra of ^{15}N-labeled PGLa or MAG2, alone or in a synergistic 1:1 mixture with the other peptide, in differently oriented lipids. Powder spectra are shown in the top row. Red, green, and blue lines indicate chemical shifts associated with the S-, T-, and I-state orientations, respectively.

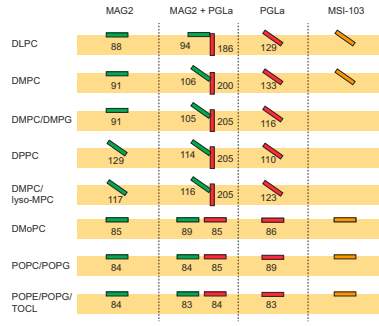

FIGURE 2 Schematic overview of the orientation of PGLa (*red*), MAG2 (*green*), and MSI-103 (*orange* (13)) in different lipids. The corresponding ^{15}N-NMR chemical shifts (in ppm) of the spectra in Fig. 1 are indicated beneath each peptide.

is in the T-state but flips into the I-state in the presence of MAG2. MAG2 is slightly more tilted than in DMPC, but it never reaches the I-state.

In contrast, in unsaturated lipids, both peptides are always in the S-state, both alone and in the presence of the synergistic partner. In POPC/POPG (9:1), the ^{15}N chemical shifts of both PGLa and MAG2, alone and in the 1:1 mixture, are between 84 and 89 ppm, clearly indicating a flat alignment on the bilayer surface. As there are no changes in chemical shift with or without the other peptide, this could indicate that there are no interactions between them, in contrast to the situation in saturated lipids. Also, in thin DMoPC bilayers (with 14 carbon atoms and a double bond), the chemical shifts of all samples show that both peptides remain always in the S-state, whether alone or mixed.

These results clearly demonstrate that the hydrophobic membrane thickness is not a critical factor for the insertion of PGLa in the presence of MAG2. In DMoPC (thinner than DMPC), there is no insertion, whereas in DPPC (thicker than POPC) insertion occurs. On the other hand, the results fully support the lipid-curvature hypothesis, which states that peptides remain on the surface in membranes composed of lipids with a negative spontaneous curvature, but are more easily tilted or inserted when the lipids have a positive spontaneous curvature (13).

In a special lipid mixture, POPE/POPG/TOCL (72:23:5), often used to mimic the composition of the inner membrane of *Escherichia coli* (15), the result is practically the same as in POPC/POPG (9:1). Also here, chemical shifts of ~84 ppm indicate that PGLa and MAG2 are always in the S-state, both alone and as a mixture. This behavior is in accordance with the curvature hypothesis, since PE and CL both have a strong negative curvature. On the other hand, when lyso-MPC is added to DMPC to increase the positive curvature, the chemical shift of MAG2 increases to 117 ppm, indicating a more tilted orientation in the membrane with enhanced curvature compared to DMPC or DMPC/DMPG, both with and without PGLa. PGLa alone gives a somewhat larger chemical shift but stays in the T-state, whereas PGLa together with MAG2 flips again into the I-state.

We can now compare the results presented here with those from our previous study on the related peptide MSI-103 (13) to find strong correlations. Fig. 2 gives an overview of all results, illustrating the peptide orientations in the different lipid systems. PGLa on its own behaves just like MSI-103 and assumes the same S-state or T-state in the same systems, in full accordance with the lipid-curvature hypothesis. MAG2 alone behaves similarly but seems to have a higher concentration threshold to flip from the S-state to the T-state. In DMPC and DMPC/DMPG, where PGLa is already in the T-state, MAG2 is still in the S-state at P/L = 1:50. However, at P/L = 1:10 (Fig. S2), MAG2 has also reached the T-state. Since MAG2 is charged at both termini, whereas PGLa and MSI-103 are amidated and thus uncharged on the C terminus, it is indeed expected that MAG2 should not start to tilt as easily as PGLa or MSI-103. The polar sector of MAG2 is also larger (Fig. S3).

When PGLa and MAG2 are mixed 1:1, their behavior correlates well with that of the individual peptides. In systems where PGLa and MSI-103 are in the S-state, the mixture of PGLa and MAG2 also remains in the S-state. Only when PGLa alone prefers the T-state does it get fully pushed into the I-state by the presence of MAG2. Thus, the model of MAG2-assisted insertion of PGLa proposed previously (12), which suggested that MAG2 would facilitate a thinning of the membrane such that PGLa would be able to insert into it, cannot be correct. We can instead conclude that only lipid systems that encourage peptide insertion per se show the MAG2-induced I-state of PGLa. The relationship between lipid shape and the tendency of peptides to insert into the membrane, as previously discussed (13), is illustrated in Fig. S4. Interestingly, common bacterial lipids like PE and CL have a negative spontaneous curvature and should thus not support peptide insertion and stable pores. However, pores could still be transient in native membranes, or other components like membrane proteins could influence the overall spontaneous curvature.

In conclusion, we propose several criteria that encourage a peptide to insert from the surface-bound S-state more deeply into the membrane (i.e., into a T-state or I-state): 1), positive lipid spontaneous curvature, which is enhanced by large headgroups and ordered lipid chains (due to saturation, but also found at low temperatures close to the gel-to-liquid-crystalline phase transition); 2), a narrow polar sector and uncharged termini of the peptide; and 3), the presence of another peptide. The other peptide might have an indirect effect by changing the membrane properties via crowding. However, for PGLa/MAG2, a distinct synergistic activity has been demonstrated, indicating more specific interactions between these two peptides. The present ^{15}N-NMR analysis shows that the two partner peptides are not aligned side-by-side as a dimer. Further solid-state NMR distance measurements will be required to clarify their detailed mode of assembly.

SUPPORTING MATERIAL

Materials, methods, supporting tables, figures, and references (16–18) are available at http://www.biophysj/supplemental/S0006-3495(13)00153-7.

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