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Peptide induced demixing in PG/PE lipid mixtures: A mechanism for the specificity of antimicrobial peptides towards bacterial membranes?

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ABSTRACT

Antimicrobial peptides attract a lot of interest as potential candidates to overcome bacterial resistance. So far, nearly all the proposed scenarios for their mechanism of action are associated with perforating and breaking down bacterial membranes after a binding process. In this study we obtained additional information on peptide induced demixing of bacterial membranes as a possible mechanism of specificity of antimicrobial peptides. We used DSC and FT-IR to study the influence of a linear and cyclic arginine- and tryptophan-rich antimicrobial peptide having the same sequence (RRWWRF) on the thermotropic phase transitions of lipid membranes. The cyclization of the peptide was found to enhance its antimicrobial activity and selectivity (Dathe, M. Nikolenko, H. Klose, J. Bienert, M. Biochemistry 43 (2004) 9140–9150). A particular preference of the binding of the peptides to DPPG headgroups compared to other headgroups of negatively charged phospholipids, namely DMPA, DPPS and cardiolipin was observed. The main transition temperature of DPPG bilayers was considerably decreased by the bound peptides. The peptides caused a substantial down-shift of the transition of DPPG/DMPC. In contrast, they induced a demixing in DPPG/DPPE bilayers and led to the appearance of two peaks in the DSC curves indicating a DPPG-peptide-enriched domain and a DPPE-enriched domain. These results could be confirmed by FT-IR-spectroscopic measurements. We therefore propose that the observed peptide-induced lipid demixing in PG/PE-membranes could be a further specific effect of the antimicrobial peptides operating only on bacterial membranes, which contain appreciable amounts of PE and PG, and which could in principle also occur in liquid–crystalline membranes.

1. Introduction

During recent years, cationic antimicrobial peptides (CAMP) have drawn much attention as a promising solution to overcome the problem of bacterial resistance. This is due to their ability to disturb biological membranes via non-specific interactions with the membrane lipid components. In spite of the large efforts undertaken to bring some of the CAMPs to the drug market [1–4], the lack of a thorough molecular-based understanding of their mechanisms of action impedes the progress in this field.

On a macroscopic level, there are two common and functionally important requirements for the antimicrobial peptide; a net cationic charge and the ability to assume an amphipathic structure, where the hydrophilic and the hydrophobic parts are oriented in opposite directions. The classical scenarios for the mechanism of action of CAMPs fall into two categories: 1) the formation of pores in lipid membranes through either transmembrane structures (for example, barrel stave and toroidal pore models) or by disturbing and thinning the lipid bilayer (for example, molecular electroporation and sinking rafts models), 2) by dissolving the membrane in a detergent-like manner (carpet model) [5–9]. In addition, other mechanisms based on the formation of lipid–peptide domains [10–14], the segregation of anionic lipids from zwitterionic ones [15–17] and the induction of non-lamellar phases [18–20] were also reported but less well studied. A peptide that works solely by one of those mechanisms is not very common and the actually observed mechanism usually depends on the lipid composition as well as on the lipid/peptide ratio. In addition, numerous antimicrobial peptides have targets inside the bacterial cell [6,21]. A key feature of the cationic peptides is their capability to distinguish bacterial from mammalian cells based on the fact that the lipid composition of their membranes is different. The outer leaflet of human erythrocytes, as a representative model for mammalian cell membranes, is composed of uncharged lipid components, mainly PC, PE, sphingomyelin and cholesterol. In contrast, the inner membrane of *Escherichia coli*, as a model for Gram-negative bacterial membranes,

Abbreviations: DSC, Differential scanning calorimetry; FT-IR, Fourier transform infrared; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine; TMCL, 1,1′,2,2′-tetramyristoyl cardiolipin; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PA, Phosphatidic acid; PS, Phosphatidylserine; CL, Cardiolipin; CAMP, Cationic antimicrobial peptide; T_m, Lipid phase transition temperature

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contains mainly PE together with substantial amounts of negatively charged phospholipids, namely PG and cardiolipin [22–25]. Among the different classes of antimicrobial peptides, the small peptides rich in arginine (R) and tryptophan (W) are of great interest due to their relatively high potency and selectivity as well as to their small size and, consequently, low cost. In these peptides, arginine provides the positive charge and the capacity for hydrogen bonding, while the hydrophobic amino acid tryptophan has a preference for the interfacial region of the membrane bilayer. Moreover, tryptophan anchors the peptides into the bilayer hydrophobic core and prolongs their attachment to the membrane [7,26].

The peptide Ac-RW (Ac-RRWWRF-NH_2) is a linear synthetic hexapeptide obtained from screening a synthetic combinatorial library [27]. C-RW was introduced by Dathe et al. through a head-to-tail cyclization of the linear sequence [28,29]. Both hexapeptides target prokaryotic membranes, yet the cyclization pronouncedly enhanced the antimicrobial activity and the selectivity towards bacterial membranes. Furthermore, both peptides were able to permeabilize lipid bilayers and their action was distinctly modulated by the lipid composition [28,29].

Using the linear and the cyclic arginine-containing model peptides, we performed DSC and FT-IR experiments on lipid vesicles and multilamellar lipid films, respectively. Our main goals were 1) to investigate the selectively of the peptides, which have the same primary structure, for negatively charged phospholipids (DPPG, DMPA, DPPS and TMCL), 2) to examine their affinity towards DPPG/DMPC and DPPG/DPPE lipid mixtures with varying surface charge density, i.e. DPPG fraction, and 3) to obtain insight into the thermodynamics of the interaction of the peptides with lipid membranes. Moreover, we intended to explore the differences in behaviour when PC is replaced with PE, which is present in the real bacterial membranes.

2. Experimental procedures

2.1. Materials

2.1.1. Peptides

The synthetic peptides Ac-RW (Ac-RRWWRF-NH_2) and C-RW (cyclo-RRWWRF) were synthesized as described before [28,29]. The peptide solutions were freshly prepared in an aqueous Tris buffer by weighing the lyophilized samples, dissolving them in buffer and diluting the samples to the required concentration. The one-letter code is used to give the sequences.

2.1.2. Lipids

1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphate-Na (DMPA) were purchased from Genzyme GmbH, Germany. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), 1,1′,2,2′-tetramyristoyl cardiolipin (TMCL) and 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-phosphoglycerol (DPPG-d_{62}) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All lipids were used as received without any further purification or modification. The concentration was calculated from the weight of the dry lipid samples (w/v).

2.2. Vesicle preparation

2.2.1. DSC

The samples were prepared in an aqueous Tris buffer (10 mM Tris/Tris–HCl, 154 mM NaCl, pH 7.4). Tris–HCl and NaCl were purchased form Sigma-Aldrich (Steinheim, Germany). The vesicles were prepared by sonication in a water bath at temperatures higher than the lipid phase transition temperature. The obtained vesicles had an average diameter of 50–100 nm as detected by DLS (data not shown). These vesicles are expected to be mostly unilamellar due to their size.

2.2.2. FT-IR

The samples were prepared in an aqueous 100 mM NaCl solution. The lipid–peptide mixtures were sonicated for 60 min at temperatures higher than the lipid phase transition temperature to equilibrate the binding of the peptides to both sides of lipid bilayers.

2.3. Methods

2.3.1. DSC

The differential scanning calorimetry measurements were carried out using a VP-DSC from MicroCal™ Inc. (Northampton, MA). From a stock solution of lipid vesicles a final concentration of 2 mM lipid was prepared. The lipid–peptide mixtures with the desired ratio were

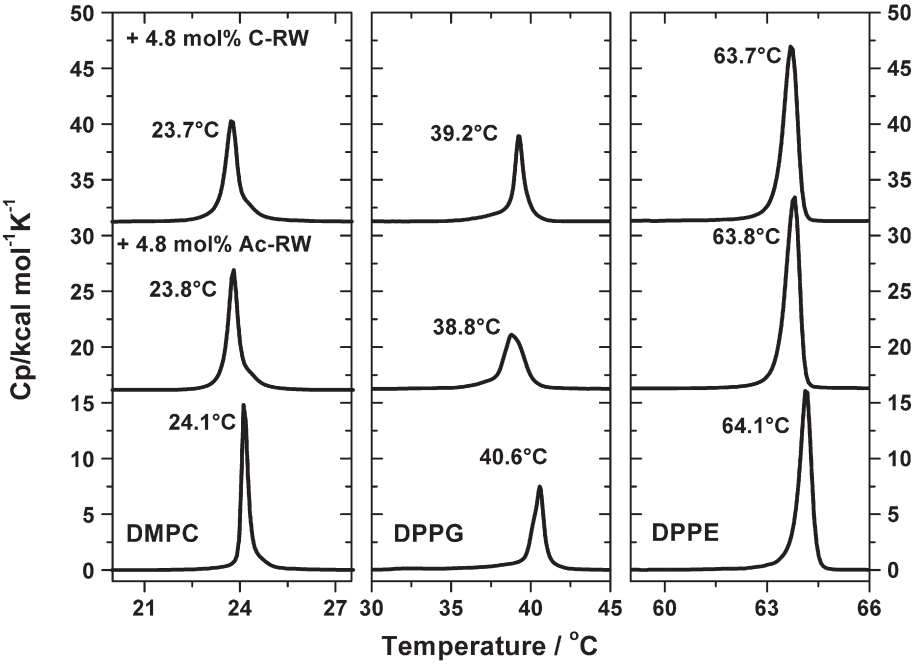

Fig. 1. DSC curves of DMPC, DPPG, and DPPE with and without added peptides C-RW and Ac-RW.

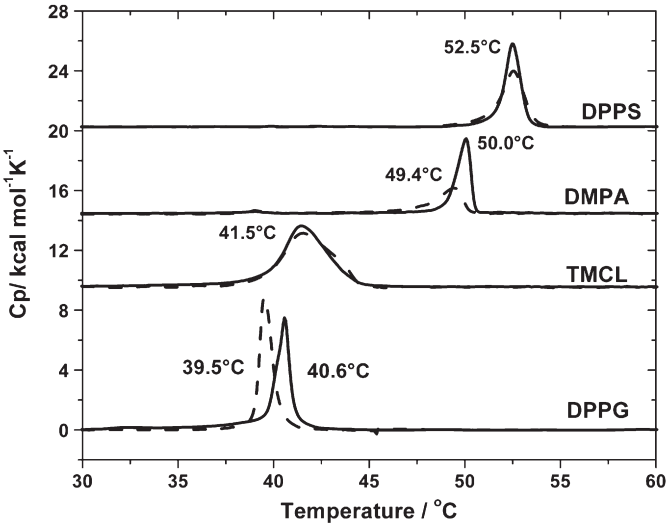

Fig. 2. DSC curves of DPPS, DMPA, TMCL and DPPG before (full lines) and after the addition of 2.4 mol% C-RW (dashed lines).

freshly pre-mixed prior to the DSC experiments. The samples were degassed for 10 min before being loaded into the sample cell. A scanning rate of 60 °C/hr was applied. The reference cell was filled with buffer. Several heating and cooling scans were performed to ensure reproducibility. All DSC thermograms shown in the figures are from the second heating scan.

2.3.2. FT-IR

The IR spectra were obtained using a Bruker Vector 22 spectrometer (Bruker GmbH, Germany) equipped with a DTGS detector. 15 µl of a 60 mM lipid/10 mM peptide mixture were placed between two CaF_2 windows with a 25 µm-thick Teflon spacer. The sample holder was temperature-controlled and at each temperature 64 scans were recorded with a resolution of 2 cm^{-1} and a zero filling factor of 2. For data processing, the Bruker OPUS FT-IR software was employed. Spectra of 100 mM NaCl solution in H_2O were used as a reference and subtracted from the sample spectra. Peak positions were determined by the second derivative method.

3. Results and discussion

3.1. DSC

Differential scanning calorimetry was utilized to inspect the influence of Ac-RW and C-RW on the main thermotropic phase transition of the anionic lipids DPPG, DMPA, DPPS and TMCL as well as of the zwitterionic lipids DMPC and DPPE. These experiments were performed to clarify the specificity of the peptides towards negatively charged phospholipids. Ac-RW (N-terminus acetylated and C-terminus amidated) and C-RW have a nominal net charge of +3 at neutral pH. Therefore, a much higher selectivity of the peptides towards negatively charged lipids over uncharged ones was anticipated due to the electrostatic attraction.

Fig. 1 shows the DSC curves of the second heating scan of DMPC, DPPG, and DPPE vesicles and of the same lipid vesicles after the addition of 4.8 mol% of the peptides Ac-RW and C-RW. The first heating scans (not shown) were always slightly different than the subsequent scans. This is caused by the fact that the peptides were added to the outside of the vesicles at room temperature and that heating the lipids through the phase transition enhanced the peptide translocation to the inner side.

The peptides shifted the main phase transition temperature (T_m) of DPPG vesicles to slightly lower values, whereas their effect on DMPC and DPPE was modest. ITC, DSC and fluorescence quenching experiments carried out before with PC, PG and PG/PC mixtures had already shown the clear preference of peptides binding to anionic vesicles [28,30,31]. The T_m of DPPG from the gel to the liquid crystalline L_α phase was detected at ~40.6 °C, which agrees well with literature data [22,32]. The T_m decreased by 1.8 °C (38.8 °C) and 1.4 °C (39.2 °C) upon adding 4.8 mol% of Ac-RW and C-RW, respectively. The linear peptide destabilized DPPG bilayers slightly more than the cyclic peptide, whereas it increased the half-width of the DPPG phase transition pointing to a decrease in the transition cooperativity as reported before [30]. Marginal changes in cooperativity were found with C-RW. The slightly weaker influence of the cyclic peptide upon the negatively charged DPPG bilayer correlates well with the slightly reduced permeabilizing activity of C-RW towards POPG vesicles as compared to Ac-RW [28].

A comparison of the influence of 2.4 mol% C-RW on the thermotropic phase behaviour of the negatively charged phospholipids DPPG, DPPS, DMPA, and TMCL is demonstrated in Fig. 2. The thermodynamic parameters of the DSC thermograms in Fig. 2 are listed in Table 1, namely the main transition temperature (T_m), the transition enthalpy (ΔH) and the half height width (HHW) as an indication of the transition cooperativity. Upon the addition of 2.4 mol % C-RW the T_m of DPPG was decreased by 1.1 °C (from 40.6 to 39.5 °C), however ΔH and HHW remained almost unaltered. The T_m of DMPA, whose negative headgroup charge is more exposed to the surrounding than that of other lipids, was also down-shifted by 0.7 °C (from 50.1 to 49.4 °C) upon the interaction with the peptide while the transition enthalpy and cooperativity were considerably reduced (Table 1). These changes in the transition of DMPA could be due to the well-known sensitivity of PA bilayers to the surrounding rather than to a specific interaction with our peptides. Unexpectedly, the T_m and ΔH of the main transition of DPPS and TMCL were barely influenced by C-RW, whereas the cooperativity of the transition (i.e. HHW) was moderately decreased. From the DSC thermograms, a clear preference of C-RW towards DPPG as compared to the other negatively charged phospholipids DPPS and TMCL was concluded. This unmistakable preference could be due to possible hydrogen bonding between the peptides and the glycerol headgroup of DPPG or due to steric effects since the size of the lipid headgroups varies, however, further investigations are required to support those notions. The effect of the zeta potential of acidic lipid vesicles on their affinity towards cationic peptides cannot be ruled out [33]. In addition, the fact that DPPG has the lowest transition temperature among the investigated anionic lipids should not be overlooked. The more selective interaction of antimicrobial peptides with PG over PS and PA [34] as well as the discriminative binding to certain lipid headgroups [35–37] was already proposed in other studies. Despite the similarities between the chemical structure of DPPG and TMCL, they interact differently with C-RW. This difference in behaviour between CL and PG molecules

Table 1
Thermodynamic parameters for the phase transition of DPPG, TMCL, DMPA and DPPS obtained by DSC as presented in Fig. 2 before and after the addition of 2.4 mol% C-RW

Lipid	T_m (°C)	ΔH (kcal $mol^{-1)}$	HHW[a] (°C)
DPPG	40.6	8.8	0.8
DPPG+C-RW	39.5	8.4	0.7
Change	−1.1	−0.4	−0.1
TMCL	41.5	11.8	2.5
TMCL+C-RW	41.6	11.8	3.1
Change	+0.1	0.0	+0.6
DMPA	50.1	4.2	0.8
DMPA+C-RW	49.4	3.2	1.5
Change	−0.7	−1.0	+0.7
DPPS	52.5	5.3	0.9
DPPS+C-RW	52.5	5.0	1.3
Change	0.0	−0.3	+0.4

[a] HHW is the half height width of the DSC peak.

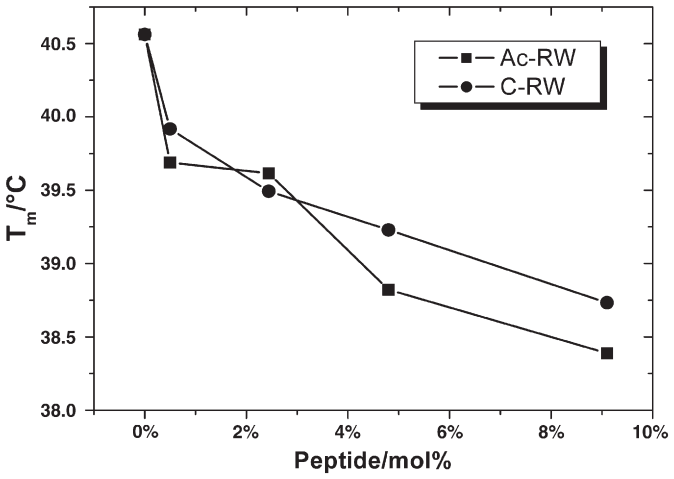

Fig. 3. Transition temperature of DPPG as a function of peptide concentration.

was reported before and is supposedly due to the higher rigidity of CL membranes leading to a lower penetration efficiency of the peptides into these membranes [38]. The possibility of the formation of cyclic intramolecular hydrogen-bonded structures in the headgroup region of CL may also play a role [39]. Furthermore, the reduced structural flexibility of the constrained C-RW hampers its deeper burial in lipid membranes, particularly when these are more ordered.

For pure electrostatic binding to PG headgroups one would expect an increase in T_m [32]. On the other hand, peptide interactions solely with the hydrophobic region of membranes lead to a perturbation of lipid chain packing and then to a reduction in the transition temperature and enthalpy [36]. The observed decrease in T_m suggests that the net effect on DPPG is primarily due to the perturbing effect on the lipid chains which overcompensates the electrostatic effect of the interaction with the headgroups.

Both, Ac-RW and C-RW, adopt an amphipathic structure when bound to a detergent micelle or to a lipid bilayer. The arginine residues and the tryptophan and phenylalanine side chains of Ac-RW are arranged on opposite sides of the backbone [30]. In the conformationally constrained C-RW, the cyclic backbone leads to the charged side chains being oriented to one side to form the hydrophilic face and a cluster of the aromatic side chains to the opposite hydrophobic part [26,40]. This arrangement allows a simultaneous electrostatic interaction of the arginine side chains with the lipid polar/charged headgroup and an insertion of the aromatic rings into the hydrophobic core of the membrane [26,28]. The scrambling of the primary sequence of C-RW was found to have a modest influence on the peptide activity and amphipathicity [41]. Thus, the destabilization induced by the peptides can be related to the perturbation of the chain packing in the DPPG bilayer by the aromatic rings of the peptides. The peptide activity was shown to be directly correlated to how deep the hydrophobic residues were buried in the membrane core [28]. For instance, the distance between the tryptophan residues of C-RW and Ac-RW and the centre of the POPC bilayer was determined to range between 9.4 to 12.4 Å, whereas their distance to the centre of the POPG/POPC 1:3 bilayer was much larger, namely 15.2 and 15.6 Å. This concurs well with the bilayer permeabilizing activity of the peptides which was 10 to 15 times higher towards POPC as compared to POPG/POPC 1:3 vesicles [28].

The DSC peaks showed no shoulders indicating a uniform distribution of the peptides in the membrane of the DPPG vesicles. Moreover, this observation suggests that the peptides can enter the DPPG vesicles, particularly when the lipid bilayers are in their fluid state, and consequently interact with the inner leaflet of the bilayer as well. This is not unrealistic as translocation across highly negatively charged lipid bilayers has recently been suggested for the cationic peptide penetratin [42]. The evidence from the DSC data for this translocation is that the first scan was always different than the subsequent heating scans. To further prove this translocation we also performed DSC scans with DPPG vesicles preloaded with the peptides (the peptides existed from the beginning inside and outside the vesicles). Only small differences were observed between the DSC curves obtained with preloaded DPPG vesicles and with the results of the former experiments (data not shown).

A more quantitative description of the destabilization of DPPG vesicles by the peptides is demonstrated in Fig. 3. A higher peptide-to-DPPG molar ratio leads to a stronger reduction, however non-linear, of the T_m with Ac-RW being slightly more efficient than C-RW. The transition enthalpy and the cooperativity remained almost unaffected over the whole tested molar ratio range. An unaltered transition

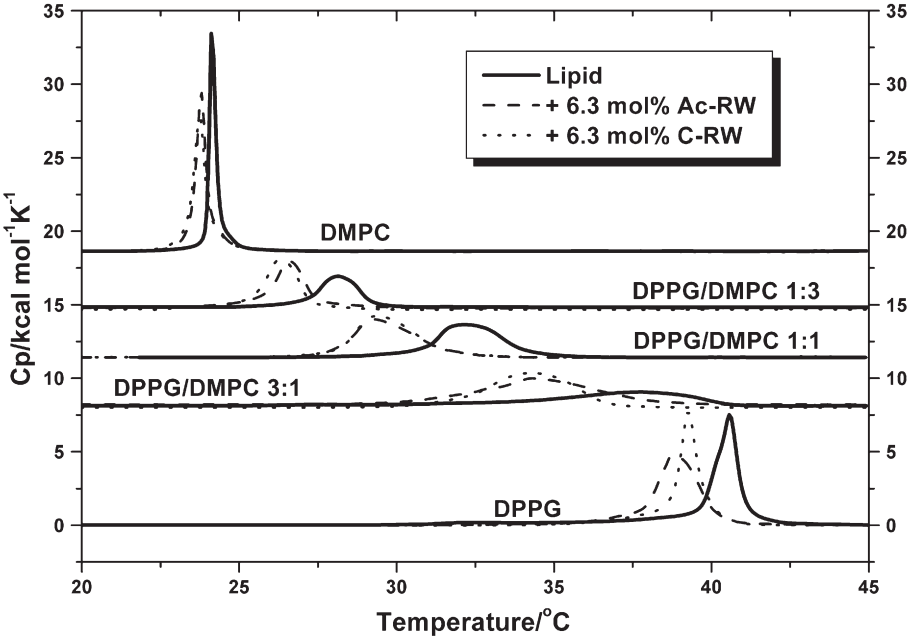

Fig. 4. DSC curves of DPPG/DMPC mixtures without (full lines) and with added peptides (dashed and dotted lines).

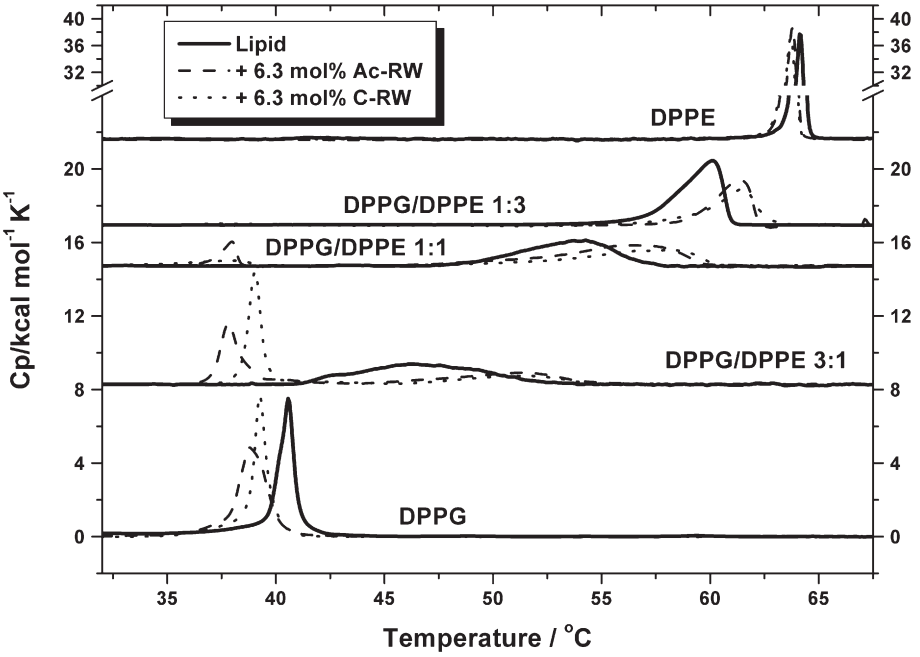

Fig. 5. DSC curves of DPPG/DPPE mixtures without (full lines) and with added peptides (dashed and dotted lines).

enthalpy for Ac-RW was also reported by Jing et al. [30]. The authors proposed that the DPPG vesicles remained intact up to 9.1 mol% peptides. Our dynamic light scattering data which show neither vesicle solubilization nor fusion after peptide binding support this assumption (data not shown).

The study of the interaction of the peptides with DPPG/DMPC and DPPG/DPPE mixed vesicles was performed to examine the effect of the surface charge density of the vesicles on peptide binding as well as to check whether there are differences between the two zwitterionic phospholipids PC and PE mixed with PG. Fig. 4 shows the DSC curves of DPPG/DMPC vesicles premixed with 6.3 mol% of the peptides. The phase transition of DPPG/DMPC lipid mixtures was shifted to a lower temperature upon premixing with the peptides (destabilization) without an indication of any demixing. Both Ac-RW and C-RW appeared to have a similar impact on the mixed vesicles. The decrease of the transition temperature is somewhat more pronounced in the mixtures than for the pure lipid vesicles, as long as there is sufficient DPPG in the vesicles to ensure adequate accumulation of the peptides at the bilayer interface due to electrostatic effects. As stated above, this phenomenon is caused by the perturbation of the lipid chain packing by the hydrophobic side chains of the peptides.

The permeabilization experiments with unsaturated PC/PG mixtures by Dathe et al. [28] showed that the activity of the peptides changes with the PG content of the mixed bilayer, being minimal at a molar POPC/POPG ratio of 1:1. Furthermore, up to a PG content of 50% the cyclic peptide C-RW was much more active than Ac-RW. This difference disappeared at a PG content higher than 50%. The DSC curves in Fig. 4 just show a down-shift of T_m but the slight differences of the effects caused by Ac-RW and C-RW, respectively, cannot be interpreted. Likewise, the discrepancies observed for different lipid compositions were not significant enough.

Our biophysical studies have been performed with lipid bilayers as a model of the lipid matrix of the cytoplasmic membrane of bacteria which is considered to be the target of most antimicrobial peptides. However, the membrane of Gram negative and Gram positive bacteria is much more complex. Thus, the selective increase of peptide activity against *E. coli* after cyclization and the inverse relationship between activity against cells and model lipid membranes indicate an important role of the outer membrane. Recent studies confirmed the activity-modulating function of the outer membrane of bacteria and underlined the importance of tryptophan and arginine residues and their relative location for a high antimicrobial effect [43].

Vesicles composed of PG and PC with various ratios are commonly used to study the effect of electrostatics, i.e. surface charge density, on the binding of cationic peptides. However, bacterial membranes do not contain PC, but rather a high amount of the zwitterionic PE in their membranes, particularly the Gram-negative bacteria. To mimic the composition of a bacterial membrane we used DPPG/DPPE mixtures to test whether the nature of the zwitterionic phospholipid has any influence on the properties of the membranes after peptide binding. We used DPPE instead of DMPE to ensure a sufficient separation of T_m of the individual lipids and a wide range of transition temperatures of the mixed lipid system. The phase diagrams of different PG/PE mixtures have been studied before and indicated complete although non-ideal miscibility in both phases [22]. This could be confirmed for our DPPG/DPPE mixtures as only one broad transition peak was observed for all mixtures without added peptide (see Fig. 5).

However, and as evident in Fig. 5, the addition of Ac-RW and C-RW to DPPG/DPPE mixtures led to the appearance of two well-separated peaks indicating lipid demixing with domain formation. The first peak at low temperature (<40 °C) corresponds to a DPPG-rich domain, where the peptide is probably bound, and the second broad peak at a higher temperature belongs to a domain enriched in DPPE with the remaining amount of DPPG and possibly some peptide. The demixing behaviour was most pronounced for vesicles with the highest DPPG content, namely the DPPG/DPPE 3:1 mixture. When the DPPG fraction in the mixture was lowered, the first peak related to the DPPG-rich domain became smaller, because the amount of available and peptide-associated DPPG in the system decreased. However, the destabilization of the DPPG-domain increased due to the higher peptide-to-DPPG ratio. Simultaneously, the remaining DPPE-rich domain becomes increasingly depleted in DPPG and the transition temperature of the second peak becomes dominated by DPPE as the main component, i.e. the T_m gets closer to that of pure DPPE.

The position of the low temperature transition that belonged to the DPPG-rich domains was always lower than that for the pure DPPG without bound peptide. Taking the data of Fig. 3, one can estimate that the low temperature peak is probably due to almost pure DPPG with a

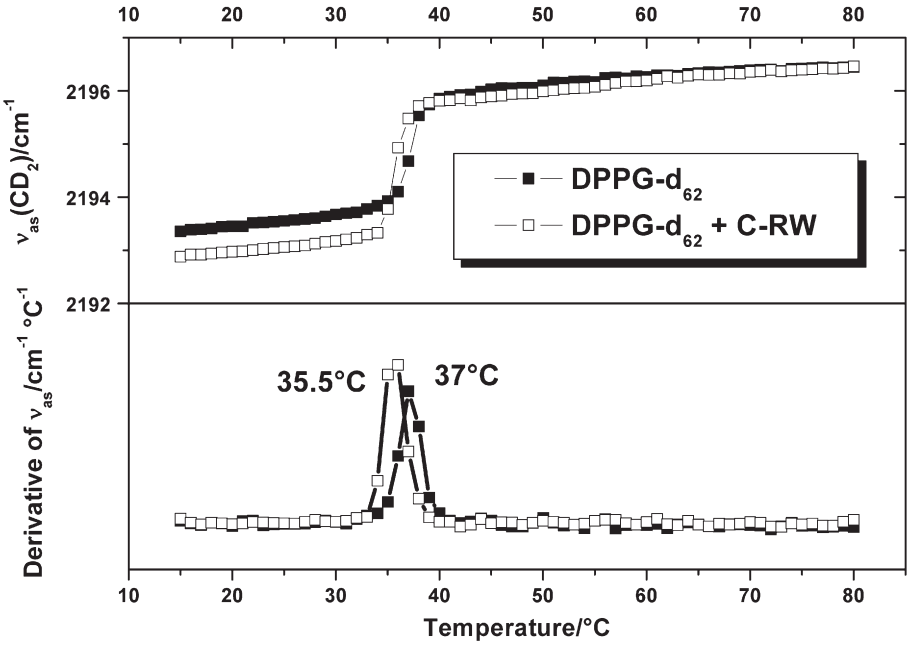

Fig. 6. Top: Temperature dependence of the wavenumber of the antisymmetric CD_2-stretching band of DPPG-d_{62} liposomes without and with (14.3 mol%) C-RW. Bottom: First derivate of the curves shown at the top.

certain amount of bound peptide. In the case of the DPPG/DPPE 3:1 mixture, the peaks of DPPG-C-RW and DPPG-Ac-RW showed up at 39 °C and 37.8 °C, respectively. For a DPPG-C-RW complex having a T_m of 39 °C, a peptide content of 6.8 mol% could be estimated using the peptide concentration dependent experiments (see Fig. 3). Again, from Fig. 3 a DPPG-Ac-RW complex with 9.1 mol% peptide content decreases the T_m of DPPG to 38.4 °C. Since the T_m of DPPG-Ac-RW complex in DPPG/DPPE 3:1 was 37.8 °C (Fig. 5), an Ac-RW content of more than 9.1 mol% is required for this effect. These estimated numbers are the lower limits for the peptide content in the DPPG domain, since the remaining DPPE domain contains residual DPPG. Independent on the exact composition of DPPG-peptide domain, it is evident that Ac-RW leads to a slightly larger down-shift of the T_m of the DPPG domains than C-RW. The DSC curves clearly indicate the following effect: in a DPPG/DPPE mixture with bound peptides separate gel phase domains of almost pure DPPG with bound peptide and peptide-poor DPPE-enriched domains exist. Upon heating, the DPPG domains become liquid–crystalline, while DPPE stills stays in the gel phase until the transition temperature of almost pure DPPE is reached. When the complete mixture is in the liquid–crystalline phase this partial demixing might persist, though the DSC curves contain no information on this process. If demixing in the liquid–crystalline phase induced by the peptides would indeed occur, this would create boundaries between domains of different composition. These boundaries would then serve as additional pathways for solutes to cross the membrane and would also destabilize the membrane. As a consequence, in addition to the usual pore formation induced by the peptides, the formation of domain boundaries due to demixing in PG/PE bilayers induced by C-RW and Ac-RW would help creating defects. Comparable data for our system on the permeabilization of unsaturated lipid bilayers of PG and PE unfortunately do not exist. Therefore, the proposed demixing in the liquid–crystalline phase remains a hypothesis and needs to be proven.

3.2. FT-IR-spectroscopy

To verify the results obtained by DSC experiments, FT-IR-spectroscopy was used to examine the influence of the peptides on DPPG, DPPG/DMPC and DPPG/DPPE multilamellar mixed-lipid films as well as to investigate the thermotropic transitions of the lipid–peptide mixtures. Here we were specifically interested in answering the question whether the peptides indeed induced demixing in DPPG/DPPE mixtures, since FT-IR can serve as a tool to follow lipid demixing [44]. The complementary and consistent nature of the information provided with FT-IR and DSC was exhibited many times in literature [e.g. [45]]. To this end, we used lipid mixtures where one of the components, in this case DPPG, contained perdeuterated acyl chains (DPPG-d_{62}). This enabled the distinction of transitions of the individual DPPG-rich and DPPE-rich domains separately. Because the frequencies of the symmetric and antisymmetric CH_2 stretching vibrations ($\nu_s(CH_2)$ at ~2850 cm^{-1} and $\nu_{as}(CH_2)$ at ~2929 cm^{-1}, respectively) are sensitive to the conformation of the lipid acyl chains, they can be used to follow the changes in the trans/gauche ratio occurring at the lipid phase transition [32,46,47]. The "melting" of the all-trans chains in the gel phase to the liquid phase, which is associated with an increase in the number of gauche conformers, is accompanied by an increase in the $\nu_s(CH_2)$ and $\nu_{as}(CH_2)$ vibrational frequencies. For the lipids with the perdeuterated chains, the corresponding $\nu_s(CD_2)$ and $\nu_{as}(CD_2)$ vibrational frequencies are at lower wavenumbers (~2090 cm^{-1} and ~2195 cm^{-1}, respectively) due to the vibrational isotope effect. In addition, the transition temperatures of lipids with perdeuterated chains are generally somewhat lower [46]. Since the T_m of DPPG-d_{62} ~37 °C is lower than the T_m of DPPG ~40.6 °C, the phase transition of the DPPG-d_{62}-containing lipid mixtures will take place at lower temperatures as compared to the DPPG-containing lipid mixtures with the same composition.

The temperature dependence of the frequency of the $\nu_{as}(CD_2)$ vibrational band in DPPG-d_{62} and DPPG-d_{62}/C-RW 6:1 (14.3 mol% C-RW) is displayed in Fig. 6. The first derivative of the curves was calculated to detect more precisely the T_m of the lipids and lipid–peptide mixtures. The T_m of pure DPPG-d_{62} was ~37 °C and decreased by ~1.5 °C upon premixing with 14.3 mol% C-RW. The interaction of C-RW and DPPG-d_{62} induced a down-shift in the CD_2 stretching vibrations below T_m, whereas above T_m the CD_2 stretching vibrations remained unaltered. The slight down-shift in the stretching vibrations is usually explained by an increase in order of the acyl chains. However, the binding of C-RW into the hydrophobic core of the lipid membrane leads to a slight decrease in T_m, indicating a less ordered

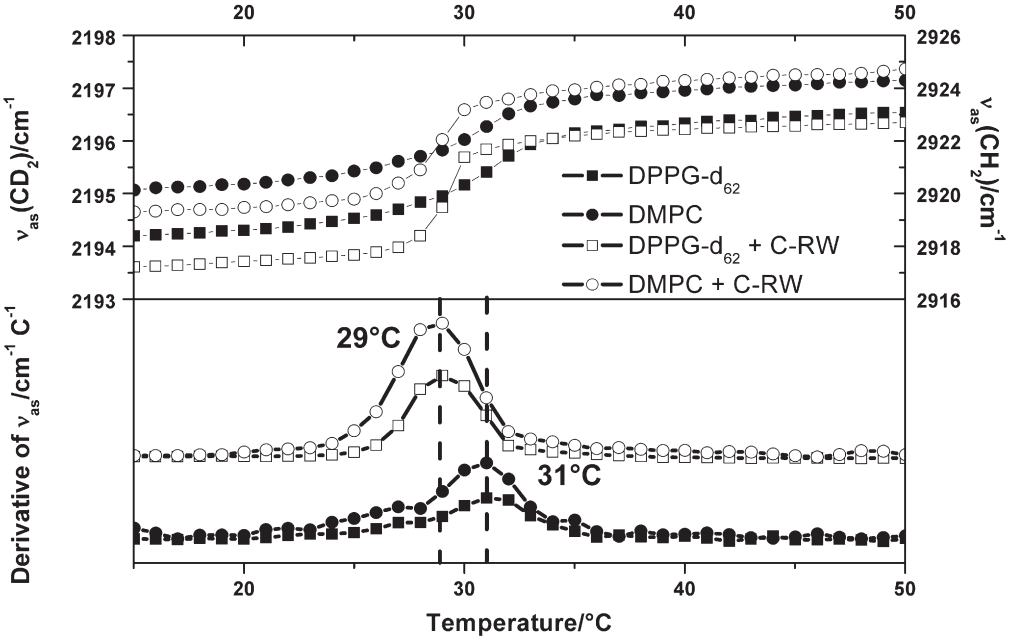

Fig. 7. Top: Temperature dependence of the wavenumber of the antisymmetric CH_2-stretching band of DMPC (right ordinate) and of the wavenumber of the antisymmetric CD_2-stretching band of DPPG-d_{62} (left ordinate) in a 1:1 DMPC/DPPG-d_{62} mixture without (full symbols) and with (14.3 mol%) added peptide C-RW (open symbols). Bottom: First derivative of the curves shown in the top diagram.

packing of the acyl chains of the lipid. Therefore, the decrease in wavenumber is probably due to other effects, which are unexplained at present but have been regularly observed upon binding of cationic peptides to negatively charged phospholipids. A probable explanation is the increase in interchain vibrational coupling caused by a reduction of rotational disorder of the chains after peptide binding [48].

The temperature dependence of the frequencies of the $\nu_{as}(CD_2)$ and $\nu_{as}(CH_2)$ vibrational bands in DPPG-d_{62}/DMPC 1:1 lipid mixture and in DPPG-d_{62}/DMPC 1:1 premixed with 14.3 mol% C-RW is illustrated in Fig. 7. The first derivative of the $\nu_{as}(CD_2)$ and $\nu_{as}(CH_2)$ bands of DPPG-d_{62}/DMPC lipid mixture, which represent DPPG-d_{62} and DMPC, respectively, showed a T_m ~31 °C. The interaction of C-RW with DPPG-d_{62}/DMPC lipid mixture decreased the T_m to ~29 °C. The similar T_m observed for the individual lipids in DPPG-d_{62}/DMPC mixture with and without C-RW suggests the homogeneity of the lipid film in both cases and no peptide induced phase separation. These results are in accordance with our DSC data shown above. Again, below the phase transition a decrease of the $\nu_{as}(CD_2)$ as well as the $\nu_{as}(CH_2)$ vibrational bands is observed. Above the phase transition temperature the frequency of the bands is not influenced by the binding of the peptide.

A different phenomenon was observed from the IR spectra of DPPG-d_{62} and DPPE in the (DPPG-d_{62}/DPPE 1:1)/C-RW 6:1 mixture (Fig. 8). The DPPG-d_{62}/DPPE 1:1 mixture has a T_m of ~54.5 °C and the temperature dependence of the CH_2- (DPPE) as well as the CD_2-band (DPPG-d_{62}) show the same transition temperature (see lower panel of Fig. 8). Upon the addition of C-RW, the frequency of the CH_2 vibrational band, which represents DPPE, showed a broad asymmetrical phase transition over a temperature range of 40–60 °C, with a peak maximum at ~57 °C. In contrast, the frequency of the CD_2 band of DPPG-d_{62} revealed a sharp transition at ~35 °C followed by a much wider transition at a higher temperature (40–60 °C) (see lower panel of Fig. 8). The presence of a phase transition at~35 °C observed in the temperature dependence of the DPPG-d_{62} band and its absence in the DPPE band supports the conclusion that demixing of the DPPG-d_{62}/DPPE lipid mixture together with the formation of a DPPG-d_{62}-C-RW complex has occurred. Again, the observations obtained by FT-IR agree well with the DSC results presented above. In the case of the DPPG-d_{62}/DPPE lipid mixture, a slight overall decrease in the frequency of the CH_2 as well as the CD_2 stretching vibrational bands is observed, in the gel and somewhat less also at high temperature in the liquid–crystalline phase of the bilayers. The usual interpretation that this is due to an increase in chain order does not hold, because then an overall increase in the transition temperature should occur. The suggestion presented above is probably also valid here, i.e. the decrease in frequency is due to increased vibrational interchain coupling, because the rotational disorder is reduced by the peptide binding.

4. Summary and conclusions

We used DSC and FT-IR to investigate the influence of a linear and a cyclic R- and W-rich antimicrobial peptide with identical amino acid sequences on the thermotropic phase transition of DPPG and other negatively charged phospholipids (DMPA, DPPS and TMCL), as well as the zwitterionic phospholipids DMPC and DPPE. Additionally, the influence of the peptides on DPPG/DMPC and DPPG/DPPE mixed vesicles with varying DPPG fraction was studied. The design of the experiments necessitated the use of phospholipids with different acyl chain lengths (C14 and C16). However, this should not have any influence on our results since our peptides due to their amphipathic nature interact mainly with the headgroup region and do not insert deeply into the lipid bilayer. Furthermore, under our experimental conditions, PG/PC and PG/PE mixtures of saturated phospholipids with an acyl chain length difference of two methylene units are completely miscible and form homogeneous bilayers in both gel and liquid–crystalline phases [22,49].

It was reported before that the structure of the linear peptide Ac-RW becomes more ordered and amphipathic upon interacting with negatively charged detergent micelles or phospholipids [30]. The cyclic peptide C-RW showed a more than tenfold increase in the antimicrobial activity over the linear Ac-RW [28,29]. The structure of C-RW was resolved using NMR [40] and its interaction with lipid bilayers described by molecular dynamics simulations [26]. The peptide adopts a constrained (rigid) conformation with two β-turns characterized by pronounced amphipathicity. This was suggested to be the reason of the enhanced antimicrobial activity and selectivity [28].

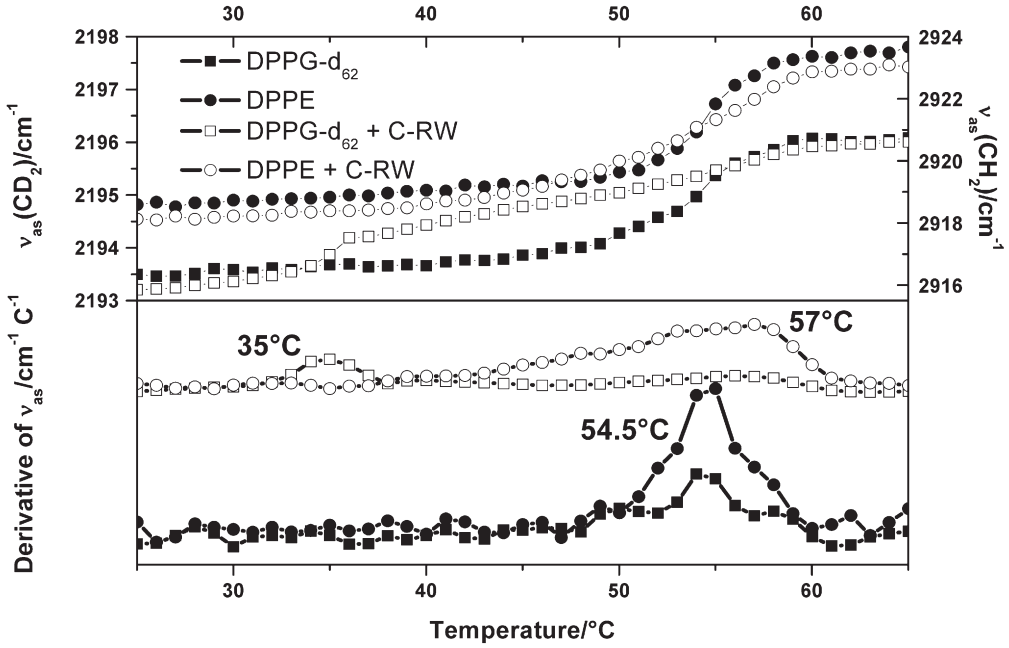

Fig. 8. Top: Temperature dependence of the wavenumber of the antisymmetric CH_2-stretching band of DPPE (right ordinate) and of the wavenumber of the antisymmetric CD_2-stretching band of DPPG-d_{62} (left ordinate) in a 1:1 DPPE/DPPG-d_{62} mixture without (full symbols) and with (14.3 mol%) added peptide C-RW (open symbols).Bottom: First derivative of the curves shown in the top diagram.

The two peptides strongly interact with negatively charged lipid membranes. The electrostatic interaction with anionic components enhances the accumulation of the peptides on the bilayer surface. However, the bilayer-influencing activity strongly depends on the nature of the negatively charged head group and the amount and properties of the zwitterionic lipids. The peptides were found to be positioned at the headgroup-acyl chain interface. Due to their zwitterionic character and the missing net charge in PC membranes the peptides are inserted deeper into bilayers composed of unsaturated PC [28]. However, the peptides are too short to form transmembrane peptide pores as in the barrel stave model. Nonetheless, our data suggest that Ac-RW but not C-RW has limited ion channel activity through the toroidal pore model (unpublished data). At a high negative surface charge density a deeper insertion into the bilayer is inhibited, but surface accumulation of a large amount of peptides is believed to disturb the bilayer by increasing the lateral surface pressure, and consequently alter its functionality [28].

The results presented here demonstrate a preference of the peptides for negatively charged lipid vesicles composed of DPPG compared to other negatively charged phospholipids, particularly DPPS and TMCL. This notion assumes a strong correlation between the peptide binding and the observed influence on the lipid phase transition. The decrease of the T_m of DPPG upon interacting with the peptides indicated that the stabilizing effect by electrostatic interactions is overcompensated by the perturbation effect on the acyl chain packing. So far, it remains unclear why the peptides prefer PG over other negatively charged phospholipids. It can only be speculated that specific hydrogen bonding effects to the PG headgroup with the glycerol moiety may play a role. This phenomenon in specificity towards PG was already reported before for other antimicrobial peptides [34].

Our results also indicate a rapid entry of the peptides into DPPG vesicles without any indication of a significant vesicle disruption and thus a homogeneous distribution of the peptides in the outer and inner leaflets.

In spite of the fact that prokaryotic membranes contain no PC, it is common that PG/PC mixtures are used as a model system for bacterial membranes. This is due to the fact that PC vesicles are more stable, easier to prepare and to handle, and that PC is zwitterionic similarly to PE. However, the two headgroups of PC and PE, respectively, have a different size and PE has the additional possibility to function as a hydrogen bond donor. Therefore the binding characteristics of peptides to PG/PE bilayers might be different.

We carried out experiments using DPPG/DMPC and DPPG/DPPE to compare the effect of peptide binding to the different lipid systems. The mixing behaviour of PG/PE mixtures was studied previously using calorimetry. In all cases non-ideal mixing was observed with negative non-ideality parameters indicating a preference for the formation of mixed PG–PE pairs [22].

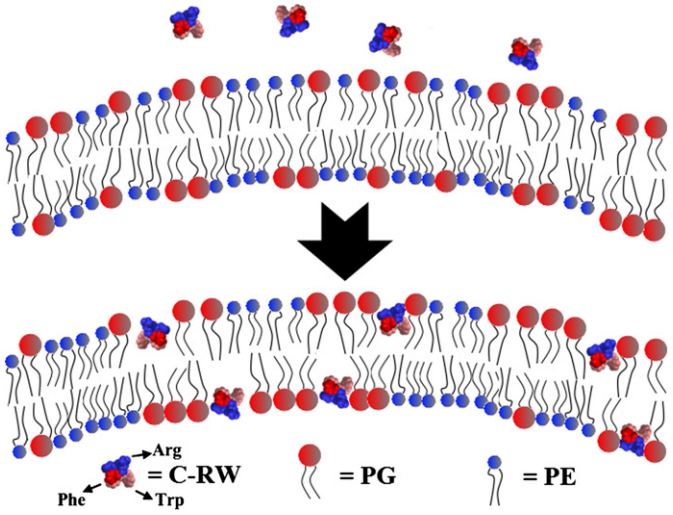

Fig. 9. Cartoon illustrating the C-RW-induced demixing in a mixed PG/PE bilayer (headgroups are represented by red and blue balls, respectively). C-RW segregates PG from PE creating defects between the formed domains. The hydrophobic amino acids Trp and Phe of the peptide are somewhat buried in the membrane core while the arginine residues interact with the PG headgroup region.

For DPPG/DMPC mixed vesicles, the addition of peptides decreased the phase transition temperature, however, no demixing was detected. On the other hand, the addition of both peptides induced a phase separation in DPPG/DPPE mixtures that resulted in the appearance of two DSC peaks; the first one due to the transition of mainly DPPG with bound peptide and the second belonging to the DPPG-depleted DPPE-enriched lipid domain. Our FT-IR results, where DPPG-d_{62} was mixed with normal DPPE to enable the separate observation of the transition of the two components in the mixture supported the DSC observations. Fig. 9 shows a cartoon illustrating the observed C-RW-induced demixing in PG/PE membranes. Less intensive peptide-induced phase segregation in DPPG/DPPE mixtures was also observed before by Lohner et al. with other peptides [14,23,36].

Further experiments are required to explain the different effects of the peptides on the miscibility in DPPG/DMPC and DPPG/DPPE vesicles. Since the peptides did not show a substantial affinity towards pure DMPC and DPPE membranes, one possible interpretation of the observed effects could be that the interactions between PG and PE in the mixture are affected due to the preference of the peptides for PG. The addition of the peptides apparently breaks up the PG–PE pair formation [22] with the consequence of demixing.

This peptide-induced segregation of different types of lipids will change the lipid environment of the peptides as well as destabilize the membrane by introducing phase boundary defects between the lipid domains. In addition, the formation of PE rich domains might destabilize the bilayers because of the high negative curvature tendency of PE lipids, particularly the unsaturated ones. Thus, the induction of phase separation in PG–PE mixtures caused by the addition of the peptides may have important consequences for their biological function and their specificity towards bacterial membranes. As the cytoplasmic membranes of Gram-negative bacteria as well as the cytoplasmic membranes of some Gram-positive bacteria contain mainly PG and PE [22–25], the possibility exists that the specificity of peptides for bacterial membranes may not rely exclusively on their higher negative surface charge but may additionally be enhanced by exploiting this mechanism of peptide induced phase separation. The correlation between the observed lipid demixing and the antimicrobial activity of the peptides needs, though, to be established. The results obtained here are valid for saturated phospholipids where the phase separation is detected by the formation of gel phase domains upon cooling. It has to be shown that the same effect occurs also in liquid–crystalline membranes. A comparable phenomenon on the interaction with fluid bilayers of mixed PG/PE and PE/CL has been reported for a flexible sequence random-polymer [15], for some isomeric alpha/beta peptides [16] and for an oligo-acyl-lysine (OAK) [17]. The phase separation in fluid mixtures might still occur but is probably on a much smaller scale and therefore difficult to prove. We just want to mention the ongoing debate about the nature of “lipid rafts”, which are small and dynamic and therefore difficult to visualize. Epand and Epand proposed that the presence of multiple cationic residues, a conformational flexibility and sufficient hydrophobicity could be a prerequisite for an antimicrobial agent to induce phase segregation of anionic lipids [10]. The observed specificity of the peptides for different headgroup mixtures containing negatively charged components suggest that this might be an additional effect for their action besides the necessity for presenting negatively charged membrane surfaces.

An important consequence of our results is that for studies of the interaction of antimicrobial peptides with model bacterial membranes, a PG–PC mixture is a poor choice. Because of the selective interactions we found in our studies, it is advisable to study PG–PE membranes as a model system for bacterial membranes, particularly Gram-negative bacteria, despite the difficulty of obtaining stable vesicle systems. In our studies using DSC and FT-IR, the aspect of vesicle stability was of minor importance, because these techniques can also be used with large multilamellar liposomes. When applying fluorescence spectroscopy the problems are, however, more severe and it might be impossible to overcome them.

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