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The efficacy of trivalent cyclic hexapeptides to induce lipid clustering in PG/PE membranes correlates with their antimicrobial activity

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ABSTRACT

Various models have been proposed for the sequence of events occurring after binding of specific antimicrobial peptides to lipid membranes. The lipid clustering model arose by the finding that antimicrobial peptides can induce a segregation of certain negatively charged lipids in lipid model membranes. Anionic lipid segregation by cationic peptides is initially an effect of charge interaction where the ratio of peptide and lipid charges is thought to be the decisive parameter in the peptide induced lipid demixing. However, the sequence of events following this initial lipid clustering is more complex and can lead to deactivation of membrane proteins involved in cell division or perturbation of lipid reorganization essential for cell division. In this study we used DSC and ITC techniques to investigate the effect of binding different cyclic hexapeptides with varying antimicrobial efficacy, to phosphatidylglycerol (PG)/phosphatidylethanolamine (PE) lipid membranes and their ability to induce lipid segregation in these mixtures. We found that these cyclic hexapeptides consisting of three charged and three aromatic amino acids showed indeed different abilities to induce lipid demixing depending on their amino acid composition and their sequence. The results clearly showed that the cationic amino acids are essential for electrostatic binding but that the three hydrophobic amino acids in the peptides and their position in the sequence also contribute to binding affinity and to the extent of induction of lipid clustering. The efficacy of these different hexapeptides to induce PG clusters in PG/PE membranes was found to be correlated with their antimicrobial activity.

1. Introduction

Facing an upcoming "post-antibiotic" era [1,2], one big challenge will be the development of new active antimicrobial substances. Promising new agents intensively studied and discussed are antimicrobial peptides (AMPs) as evolutionary ancient molecules to control the coexistence of microbes and complex multicellular organisms [3]. Besides the antimicrobial peptides that show an intracellular mode of action, a huge number of antimicrobial peptides are discussed to target the cell membrane. [4,5] For the latter ones many different models have been suggested for the mechanism of antimicrobial action, most of them trying to describe the experimentally observed higher permeability of the lipid membranes after peptide binding. Each model can explain the effects found for the action of only some of all peptides, many effects thus being unexplained. Wimley and Hristova [6] already referred to this fact in their review article by citing the poem by John Godfrey Saxe "The blind men and the elephant" underlining that a comprehensive explanation of antimicrobial peptide action is still missing. As some peptides show a high antimicrobial effect but only low enhancement of permeability of lipid membranes in comparison to other AMPs [7], clustering of lipids after peptide binding has been discussed as a possible further mechanism of antimicrobial action [8] and has been reported for cyclic [9–11], α-helical [12,13] and β-turn [14] AMPs as well as cell penetrating peptides with antimicrobial activity [15]. The induction of lipid clustering may be the decisive initial process for further steps in a cascade of events, such as perturbation of lipid reorganization in cell division or changes in the environment of membrane proteins relevant for cell division influencing their functionality [16].

Up to now computational analyses and synthetic combinatorial libraries [17–19] of natural occurring antimicrobial peptide structures succeeded to predict sequences of small artificial model peptides with high antimicrobial potency that found their way into clinical trials [20]. The most common feature of AMPs is their amphipathic structure, therefore the synthesized small artificial peptides contain mostly the charged residues arginine, histidine or lysine and the hydrophobic amino acids tryptophane, leucine and phenylalanine [21]. Dathe et al. showed that short cyclic peptide sequences show a higher antimicrobial activity and selectivity then the linear analogues [22]. Additionally, the higher stability towards trypsin [23] makes them attractive pharmaceuticals.

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The cyclic peptide c-RRWWRF has been shown to induce demixing in DPPG/DPPE bilayer membranes [11]. However, the driving forces leading to peptide induced lipid segregation still remain unclear. In our study we wanted to investigate the efficacy of cyclic trivalent peptides with different sequences and known different antimicrobial activities on the demixing of DPPG/DPPE bilayers. We used a cyclic hexapeptide with alternating arginine and tryptophane sequence and investigated the effect of replacing the cationic arginine by lysine and the hydrophobic amino acid tryptophane by phenylalanine. We additionally studied the effect of the sequence of the amino acids in these cyclic peptides. For a comparison we additionally investigated the effect of c-KKWWKF, the lysine analogue of the previously used c-RRWWRF and c-RRRWFW, which showed the highest antimicrobial activity of the sequences tested. Fig. 1 shows the primary sequence of the peptides investigated with their respective minimal inhibitory concentrations towards *Escherichia coli* and *Bacillus subtilis*. MIC values for c-RRRWWW and c-RRRWFW towards other bacterial strains are given by Speck et al. [24].

We determined the efficacy of these different peptides for inducing lipid clustering by analysing the phase transition of DPPG and DPPG/DPPE bilayers utilizing DSC (differential scanning calorimetry) and ITC (isothermal titration calorimetry) to study their binding properties. So far, peptide induced demixing was mostly explained by being caused by charge interactions, and thus being mainly dependent on the number of charges [15]. However, already our previous work indicated that lipid clustering induced by cationic peptide binding is lipid specific, as mixtures of DPPG with DPPC did not show phase separation after peptide binding (9). Lipid specific in the sense used here means that the composition of the mixture is essential, i.e. not only the chemical structure of the negatively charged lipid, but also the structure of the other lipid component. It could be shown that the binding of c-RRRWFW to mixtures of cardiolipin (CL) with POPE, lipids with preferred negative curvature, is much more efficient than to PC–PG mixtures [16].

In this study we used a DPPG/DPPE mixture as in the previous study with c-RRWWRF, where peptide induced demixing was clearly visible, [11] but investigated the impact of different sequences in the cyclic peptide. Although an earlier article reported no effects of sequence variations on the lipid clustering ability [26], we will show here that the amino acid sequence in trivalent cyclic peptides has a definite impact on the ability to induce lipid segregation.

2. Materials and methods

2.1. Chemicals

1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Genzyme (Neu-Isenburg, Germany). The synthetic cyclic peptides c-RWRWRW, c-RRRWWW, c-RRRWFW, c-KKWWKF were synthesized as described before [7,22]. The compounds were characterized using mass spectrometry and RP-HPLC. The determined mass [M + H] corresponds to the calculated value with a variation of maximal 1 g/mol. The HPLC purity was always better than 95%.

The peptides c-RFRFRF and c-KWKWKW were purchased from Genecust (Dudelange, Luxembourg) with a purity >98% as shown by HPLC.

2.2. Sample preparation

All samples were prepared in 20 mM phosphate buffer (pH 7.2) with 100 mM NaCl. The peptides were weighed after lyophilisation, dissolved in phosphate buffer solution, diluted to the required concentration, and degassed in a vacuum chamber before further use.

Binary lipid mixtures were prepared by dissolving each single lipid in chloroform/methanol 2:1 and mixing appropriate volumes of each stock solution. The organic solvent was removed by applying a gentle N_2 stream. The remaining lipid mixture was dried overnight under vacuum at 40 °C. The lipids were dispersed in the required buffer and extruded 17 times through a 100 nm diameter polycarbonate filter at a temperature higher than the phase transition temperature of the lipid by using an Avanti Mini Extruder (Avanti Polar Lipids, Alabaster, Alabama, USA). The vesicle size was checked by dynamic light scattering (DLS) using an ALV-NIBS/HPPS DLS instrument (ALV-Laser Vertriebsgesellschaft, Langen, Germany). Single lipid dispersions were extruded the same way after weighing and dissolving them in buffer.

2.3. Differential Scanning Calorimetry (DSC)

The effect of the cyclic peptides on the phase transition temperature of phosphatidylglycerols and their respective mixtures with phosphatidylethanolamines was investigated using a VP-DSC microcalorimeter (MicroCal, Northhampton, Massachusetts, USA). Extruded vesicles of DPPG and the respective mixtures with DPPE were mixed with aliquots of peptide solution to obtain the desired lipid to peptide ratio and a lipid concentration of 1 mM. The concentration was checked by comparing the observed DSC scans of the lipid suspension without added peptide with known values. [27] The determined transition enthalpies varied within ±3% of the published values. The applied scanning rate was 60 °C/h. The reference cell was filled with the appropriate aqueous phosphate buffer solution. Several heating and cooling scans were performed to check for reproducibility. The first heating scan was always different due to the fact that the sample was not yet equilibrated when the peptides were added at room temperature to the DPPG or DPPG/DPPE vesicle solution. During the heating and cooling scans the peptide could cross the membrane and equilibrate on both sides of the vesicular membrane. Once this had happened, further heating and cooling scans were identical and reproducible. In the figures only the last heating scan is displayed.

2.4. Isothermal Titration Calorimetry (ITC)

To determine the thermodynamic parameters of the peptide lipid interaction, ITC measurements with a MicroCal VP-ITC microcalorimeter (Microcal, Northhampton, MA) were performed. The lipid vesicle suspension was either titrated into a peptide solution in the cell or vice versa. For instance, a 62 μM peptide solution was added in 10 μL aliquots into the reaction cell (V = 1.4591 mL) containing a 2 mM lipid

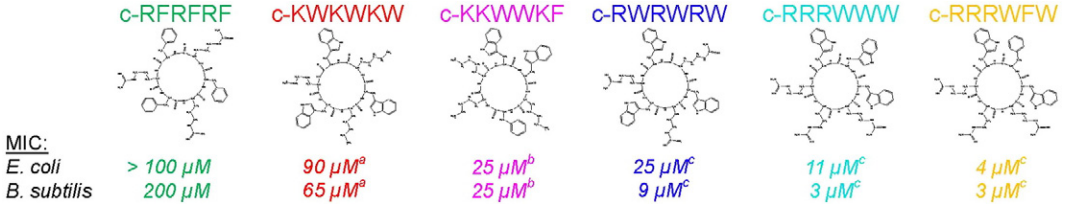

Fig. 1. The cyclic trivalent hexapeptides used in this study with their respective minimal inhibitory concentration [23,25,7]. The effect of c-RFRFRF on growth of *E. coli* and *B. subtilis* is was determined as described before. The colour coding used for the different peptides is the same as used below in the presentation of data.

dispersion with an injection duration of 20 s. The equilibration time between each titration step was 15 min. A first titration of 2 μL was disregarded to ensure that a premixing of both solutions during the equilibration time does not affect the first titration step. During the experiment the stirring speed of the injection syringe was 286 rounds per minute. The reversed titration experiments were performed similarly. The respective concentrations of peptide and vesicle solution, respectively, are given in the figure legends.

3. Results and discussion

3.1. DPPG

3.1.1. Differential Scanning Calorimetry (DSC)

The effect of different hexapeptides on the DPPG main phase transition is shown in Fig. 2. The overall effect of the binding of the peptides to pure DPPG bilayer vesicles is small. The transition temperature varies from 39.7 °C to 40.3 °C with the transition of pure DPPG vesicles being observed at 40.1 °C. This is in agreement with previous observations for other peptides with several adjacent aromatic and charged side chains, for instance c-RRWWRF [11]. Also the transition enthalpy of 10 kcal mol^{-1} for DPPG varies only slightly by about −0.9 kcal mol^{-1} for DPPG interacting with c-RRRWFW and by +0.9 kcal mol^{-1} for DPPG interacting with c-RWRWRW. The binding of the peptide with the alternating sequence containing arginine and phenylalanine (c-RFRFRF) leads to a slight increase of the phase transition temperature indicating gel phase stabilization. The peptide containing lysine and tryptophane in an alternating sequence (c-KWKWKW) does not affect the phase transition temperature at all. The remaining peptides shift the phase transition temperature to slightly lower temperature as it has also been reported for the cyclic antimicrobial peptide polymyxin B [28]. These effects do not vary significantly in the peptide concentration range studied here.

Although the cationic peptides compensate the anionic lipid charges the effect on lipid phase transition is only marginal, indicating an interplay of counteracting effects like charge compensation, headgroup hydration and dehydration as well as frustration of lipid alkyl chain interaction by penetration of the hydrophobic side chain residues into the hydrophobic bilayer region and the effect of different charge distances [29]. Comparing c-RFRFRF with an alternating arginine phenylalanine sequence with c-RWRWRW with alternating arginine tryptophane amino acids, the downshift in T_m observed for the latter indicates an insertion of tryptophane residues into the hydrophobic chain region [30,31]. This effect counteracting the charge compensation seems to be more pronounced with increasing hydrophobic volume and even more pronounced with increasing amphipathicity, indicating that the amphipathic peptides penetrate deeper into the hydrophobic chain region. This has been suggested before based on investigations of c-RWRWRW, c-RRRWWW and c-RRRWFW lipid vesicle interaction using circular dichroism [7].

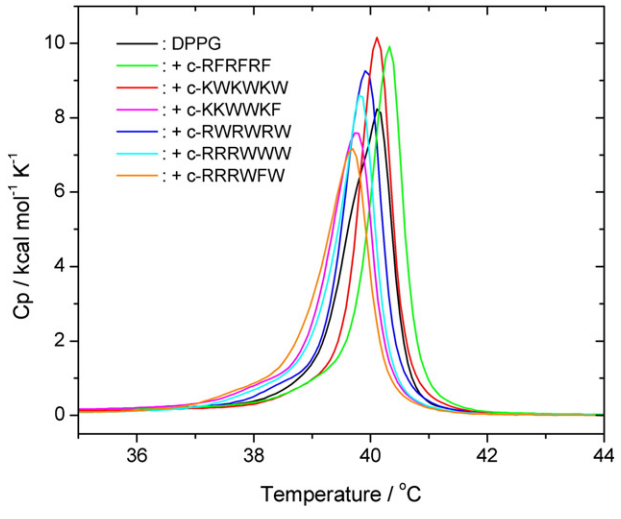

Fig. 2. DSC thermograms of pure DPPG and DPPG with cyclic hexapeptides in a 20:1 lipid/peptide ratio.

3.1.2. Isothermal Titration Calorimetry (ITC)

The ITC results for the binding of the peptides to DPPG at 20 °C are shown in Fig. 3. The lipid vesicle suspension was titrated into the peptide solution. The process is clearly exothermic for c-RWRWRW and slightly endothermic for c-RFRFRF and c-KWKWKW binding to a DPPG membrane in the gel phase. In the liquid crystalline state of the lipid the binding processes of all three peptides are exothermic (not shown). The stoichiometry factor for binding is about three lipids per peptide, indicating that the number of peptide charges determines the stoichiometry of binding, as binding is driven by electrostatic interactions.

The determined binding constants at 20 °C are $2 \cdot 10^5$ M^{-1} for c-RFRFRF, $5 \cdot 10^5$ M^{-1} for c-KWKWKW and $8 \cdot 10^5$ M^{-1} for c-RWRWRW. They are slightly lower but in the same range as binding constants determined for binding of c-RRRWFW to POPG/POPE membranes [16]. Earlier studies on electrostatic binding showed that the binding constant increases tenfold with each additional charge, and that the observed K-values for trivalent molecules such as R_3, K_3 [32] or spermidine [33] binding to a PG membrane are ca. 10^3 M^{-1}. The two orders of magnitude higher K-values for the cyclic peptides clearly indicate that the hydrophobic residues also contribute as a driving force for binding to lipid vesicle membranes.

The binding enthalpy for c-RFRFRF and c-KWKWKW shows no temperature dependence neither for binding to the gel nor the liquid

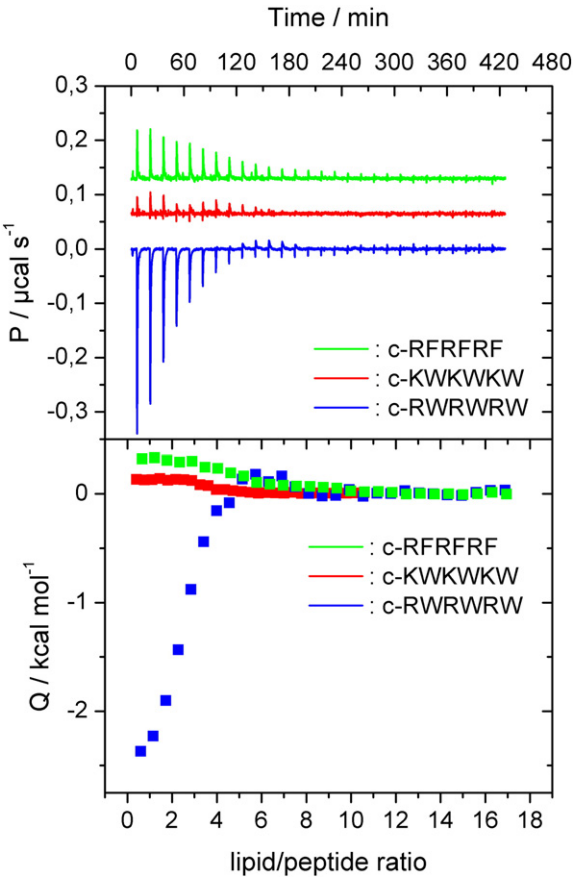

Fig. 3. Top: ITC titration curves at 20 °C of 1.6 mM DPPG vesicle suspension titrated into 20 μM c-RFRFRF, 1 mM DPPG titrated into in 20 μM c-KWKWKW and 0.8 mM DPPG titrated into 10 μM c-RWRWRW. All solutions contained 20 mM phosphate buffer (pH 7.2) with 100 mM NaCl. Bottom: Heat of binding Q in kcal mol^{-1} of lipid vs. lipid-peptide ratio.

crystalline phase (data not shown). This might be a result of compensation of heat effects arising from the two underlying processes. For DPPG titrated to c-RWRWRW two separate processes are observed, a first exothermic with a stoichiometry of ca. 3 followed by a slightly endothermic processes with a higher stoichiometry. The first process shows a negative temperature dependence, i.e. a negative $\Delta_R C_p$, meaning that with increasing temperature the binding enthalpy becomes more exothermic (data not shown). This indicates dehydration of hydrophobic surfaces. A similar temperature dependence was also found for the binding of cations compensating the PG lipid charge and therefore leading to better lipid chain interaction and reduced water content in the hydrophobic bilayer region [34], thus dehydration of hydrophobic surfaces of lipids is the cause. Also, for poly-L-arginine binding to DPPG it was found that at higher temperature the binding enthalpy became more exothermic. In this case, however, the negative $\Delta_R C_p$ is due to dehydration of the hydrophobic amino acid side chains of arginine, when they are inserted into the lipid bilayer. [31,35] That only for c-RWRWRW with alternating amino acids this negative $\Delta_R C_p$ and also much more exothermic heats of binding were observed might be a result of a the cation- π interaction between the guanidinium moiety of arginine with the indole ring of tryptophane [36] leading to a better insertion into the hydrophobic region of the bilayer with consecutive dehydration of the amino acid side chains. Comparison with the binding constants observed for c-RRRWFW shows that for the cyclopeptides with alternating charged and hydrophobic amino acids the binding affinity is lower. This might be due to the undefined ring structure of c-RWRWRW, whereas in c-RRRWFW the ring structure is stabilized by H-bonding [25]. The stabilization of the ring by H-bonding increases the hydrophobicity of the peptide and therefore the affinity for insertion into the hydrophobic interior of the membrane [37]. The structures of some of the cyclic hexapeptides in solution or when bound to micelles have been determined by NMR. These structures may differ as the side chains can adapt to an optimal conformation when bound to a negatively charged micellar surface. Also a β-sheet structure can in principle be formed. [38,39] The peptide conformation when bound to a lipid bilayer is not known at present, but it can be expected that a conformation is formed where the electrostatic and hydrophobic interactions are optimized.

3.2. DPPG/DPPE mixtures

We have shown before that the hexapeptide RRWWRF in the linear and the cyclic form induces a lipid demixing in lipid mixtures containing PG and PE but not in PG-PC mixtures [11]. This demixing is therefore not a simple consequence of the binding of the cationic peptides to the negatively charged PG, but can only be explained by assuming more specific interactions of the peptides with lipid headgroups in the mixture and on the tendency of the lipids for preferring negative intrinsic curvature [16]. With the peptides studied here, we found similar effects and tried to quantify the ability of cyclic hexapeptides of inducing lipid clustering depending on the amino acids and their sequence.

3.2.1. Differential Scanning Calorimetry (DSC)

The effect of various concentrations of the six different cyclic hexapeptides on the phase transition of a DPPG/DPPE mixture is shown in Fig. 4. The lipid to peptide ratio was varied between 75:1 and 10:1. All six cyclic peptides were able to induce a phase separation in DPPG/DPPE mixtures even at very low concentration, i.e. high lipid to peptide ratio. This is evident from the appearance of a transition peak at low temperature around 40 °C corresponding to the phase transition of almost pure DPPG clustered by the binding of the peptides. The temperature of this transition remains unchanged with increasing peptide concentration, except for the two peptides c-RRRWWW and c-RRRWFW at high peptide concentration, i.e. low lipid-peptide ratio (10:1). It is also evident from the DSC curves that the enthalpy of the lower transition increases with increasing peptide concentration, supporting the assumption that the amount of clustered DPPG is related to the amount of

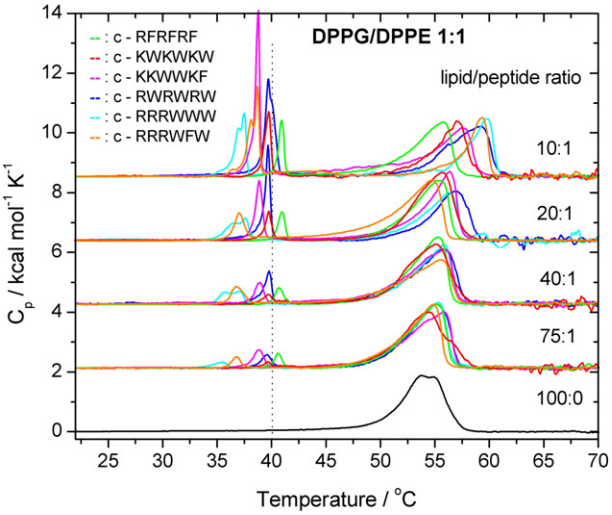

Fig. 4. DSC thermograms of vesicles of DPPG/DPPE (1:1) (black) and DPPG/DPPE (1:1) after addition of the cyclic hexapeptides (lipid-peptide ratios are indicated). The dotted black line indicates the phase transition temperature of pure DPPG.

bound peptide. The temperature of the second transition peak, corresponding to the transition of the remaining DPPE still mixed with some DPPG, increases with increasing peptide concentration, because the mixture is reduced in the lower melting component DPPG, due to the clustering of DPPG induced by the peptides.

The phase transition temperature of the clustered DPPG domains in the mixed DPPG/DPPE bilayers varies much stronger (from 35.4 °C for the DPPG-c-RRRWWW cluster to 40.9 °C for the DPPG interacting with c-RFRFRF) than for pure DPPG interacting with the different peptides (see Fig. 2). For all peptides except c-RFRFRF a phase downshift of the transition temperature of the clustered DPPG domains can be observed. Only for the binding of c-RFRFRF the phase transition temperature of the clustered DPPG is shifted to slightly higher temperature compared to the pure DPPG interacting with c-RFRFRF. The temperature difference is most pronounced in cases of interaction with the most amphipathic hexapeptides c-RRRWWW ($\Delta T = -4.3$ °C), c-RRRWFW ($\Delta T = -3.0$ °C) and c-KKWWKF ($\Delta T = -0.9$ °C). This indicates that the penetration of the cyclic peptides into the hydrophobic membrane region induces a perturbation of the lipid chain packing. The perturbation of the bilayer packing induced by c-RRRWFW and c-RRRWWW correlates with their high effective hydrophobicity and amphipathicity as determined by reversed phase HPLC [23,40].

Fig. 5 (top) shows the transition enthalpy of the low temperature peak assigned to the DPPG transition as a function of lipid-peptide ratio. From this plot the amount of clustered DPPG induced by the binding of the peptides can be roughly estimated assuming that the molar transition enthalpy of the lipid in the DPPG-peptide cluster decreases only slightly compared to pure DPPG (see DSC thermograms in Fig. 2). The transition enthalpy of the peak at low T_m is then an indication for the amount of clustered DPPG. In case of the peptides with alternating sequence the percentage of DPPG clustered into a lipid-peptide domain at a 10:1 lipid-peptide ratio, is only about 30% for c-RFRFRF, 40% for c-KWKWKW but around 80% for c-RWRWRW. Taking a possible decrease of the transition enthalpy of the DPPG-cluster into account, these numbers would slightly increase.

The temperature of the phase transition maximum of the resulting DPPG-depleted domains is in good agreement for c-RFRFRF and c-KWKWKW with the expected values. For the DPPG/DPPE-c-RWRWRW 10:1 mixture the phase transition maximum at 59 °C for the remaining DPPE enriched mixtures is slightly below the expected 62 °C for a pure DPPG/DPPE mixture with the same composition, i.e. 8:2 [27]. However, this marginal deviation is probably within the precision of the experiments.

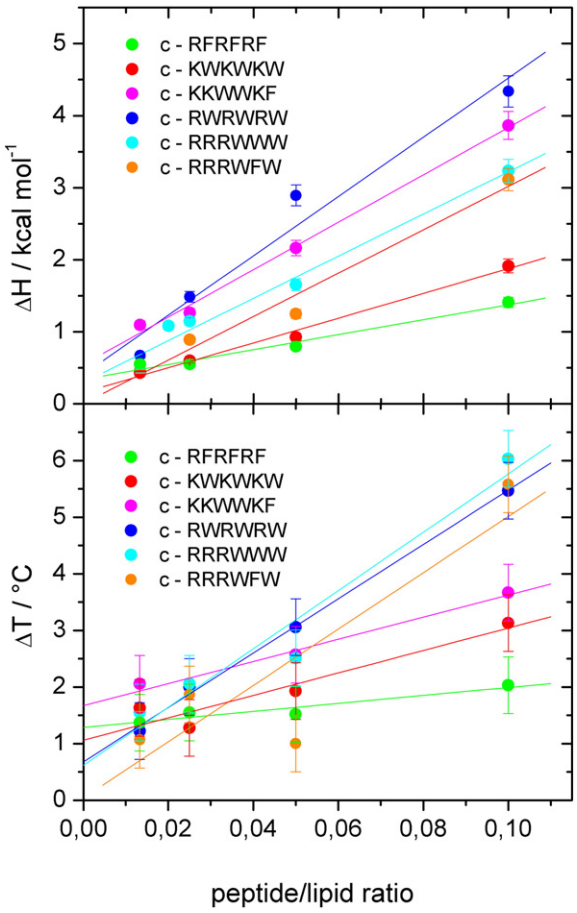

Fig. 5. Top: Transition enthalpy of the peak at lower temperature. Bottom: Difference in phase transition temperature (peak value) of the DPPG/DPPE 1:1 mixture and the upper phase transition peak of thermograms of DPPG/DPPE 1:1 mixture interacting with cyclic peptides.

Fig. 5 (bottom) shows the shift in transition temperature of the upper peak as a function of the peptide-lipid ratio. This temperature shift should correlate with the transition enthalpy of the clustered DPPG domains, i.e. the larger the temperature shift the higher the phase transition peak at low temperature due to the clustered DPPG domains. The highest temperature upshift is observed for c-RRRWWW and c-RRRWFW (Fig. 5, bottom). However, the values of the transition enthalpy of the DPPG cluster are lower after binding of these peptides than expected (see Fig. 5, top). We therefore conclude that the hydrophobic peptide residues perturb the alkyl chain interaction and lead to a reduction of the phase transition enthalpy of the clustered DPPG in the mixture, though the amount of clustered lipid is the highest for the most amphipathic peptides c-RRRWWW and c-RRRWFW as indicated by the temperature shift. Thus the shift in temperature of the upper transition peak seems to be a more reliable indicator for the efficacy of the peptides to induce phase separation. The order of the ability to cluster DPPG in a DPPG/DPPE mixed membrane, deduced from this temperature upshift of the high temperature phase transition at a 10:1 lipid-peptide ratio (Fig. 5, top), is c-RRRWWW > c-RRRWFW > c-RWRWRW > c-KKWWKF > c-KWKWKW > c-RFRFRF which resembles with good agreement the different antimicrobial activities of the peptides towards *B. subtilis* (see Fig. 1).

3.2.2. Isothermal Titration Calorimetry (ITC)

The DSC experiments clearly showed that binding of the different cyclic peptides always lead to phase separation in a DPPG-DPPE mixture with almost pure DPPG with bound peptides separating from the rest of the lipids. The efficacy of the peptides to induce this demixing has a similar sequence as their antimicrobial activities.

To further quantify the effect of demixing after binding we investigated the induction of lipid domains at constant temperature by titrating either peptides to lipid vesicles or vice versa using ITC. Of particular interest was the question whether demixing takes place immediately with the first bound peptides or whether a certain threshold ratio was needed. The heats of reaction in titrations of the peptide c-RRRWFW to DPPG/DPPE (1:1) vesicles at different temperatures are shown in Fig. 6.

At 43 °C without bound peptides the DPPG/DPPE mixture is still in the gel state (see Fig. 4). After injection of the peptide solution exothermic peaks indicating binding can be observed. The exothermic binding peaks persist till a lipid-peptide threshold ratio of ~500:1 (peptide: lipid = 0.002) is reached and then a much larger exothermic peak is seen. Titrating additional peptide to the vesicles leads to the appearance of endothermic peaks. This is obviously due now to a formation of lipid-peptide clusters that have to undergo a P_β–L_α phase transition at this temperature, i.e. at 43 °C part of the vesicles membranes are now in the liquid-crystalline state as shown by the DSC curves in Fig. 4.

Below a temperature of 43 °C, i.e. below all phase transitions observed by DSC, only exothermic heats of binding are observed. Again, at certain thresholds values of 1000:1 (10 °C, 17 °C) and 500:1 (25 °C) larger exothermic peaks are seen. These larger peaks can be interpreted by assuming that at these peptide/lipid ratios permeabilization of the vesicles takes place, so that inside binding can also occur. The high exothermic reaction heat is then due to the cumulative heat effect of binding peptides to the inside of the vesicles. Also the release of curvature stress leading to morphological changes [41] induced by the initial

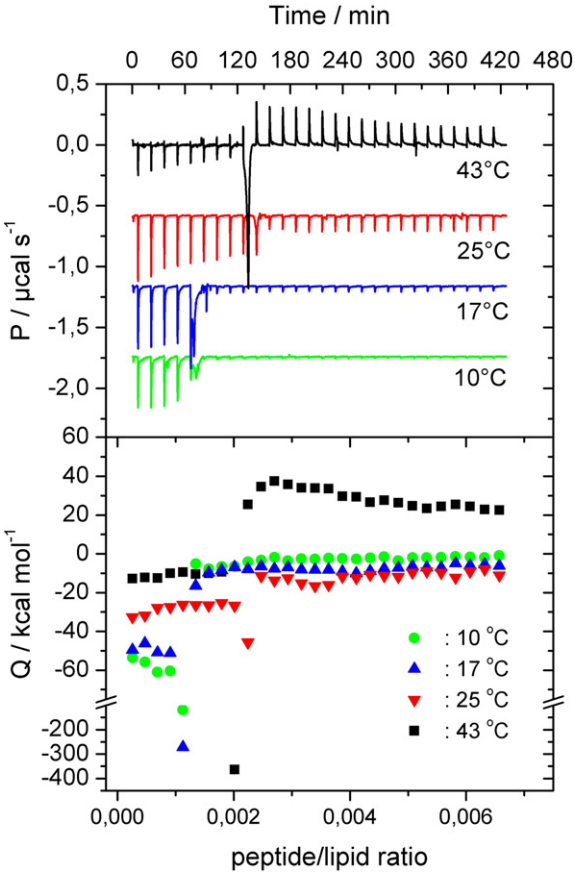

Fig. 6. Top: ITC titration curves (heating power vs. time) of 62 µM c-RRRWFW into 2 mM vesicle suspension of DPPG/DPPE (1:1) at different temperatures for lipids in the gel phase. Bottom: integrated heats calculated per mol of injected peptide vs. peptide-lipid ratio.

binding of peptides to the outer monolayer could be a process contributing to the large exothermic reaction heat.

Comparison of the binding properties of different cyclo-peptides leads to the following observation. The exothermic reaction heats observed for the different peptides seem to be correlated with their ability to induce lipid-peptide clusters as seen by DSC. The peptides c-RRRWWW and c-RRRWFW showed the strongest exothermic events whereas c-RWRWRW and c-KKWWKF showed only a moderate exothermic reaction peak. In these cases the highest peak was observed at 10 °C with −30 kcal per mol c-RWRWRW and −15 kcal per mol c-KKWWKF, respectively. Almost no exothermic reactions were observed at any temperature for c-RFRFRF and c-KWKWKW.

The dependence of the reaction enthalpy of initial peptide binding to gel phase bilayers on temperature is shown in Fig. 7. The binding enthalpy becomes less negative with increasing temperature. The slope of the temperature dependence of the binding enthalpy $\Delta_R C_P$ yields information on changes in hydration. Positive slopes correspond to a dehydration of hydrophilic and hydration of hydrophobic regions. Unfortunately, it is not easy to separate both contributions. The effect of headgroup dehydration on $\Delta_R C_P$ by was determined for Ca^{2+}, Sr^{2+} and Mg^{2+} binding to be approximately 10 cal mol^{-1} K^{-1} [34]. The hydration of a methylene group gives a $\Delta_R C_P$ value of ca. 15 cal mol^{-1} K^{-1} [42]. The reaction heats for c-RFRFRF binding to the DPPG/DPPE membranes are very small and also the $\Delta_R C_P$ value is very low, 4 cal mol^{-1} K^{-1}. For c-RRRWFW binding the highest $\Delta_R C_P$ value was found, 1450 cal mol^{-1} K^{-1}. This high value indicates that after binding extended hydrophobic surfaces are now accessible to water, probably caused by a perturbation of the bilayers by the bound peptide. The penetration of the peptides into the lipid alkyl chain region seems to induce a local exposure of the lipid alkyl chains to water. The $\Delta_R C_P$ values for the binding enthalpy of the other peptides lie in these two extremes. One can conclude that the ability to disturb the lipid chain packing by peptide insertion corresponds to the ability to induce lipid-peptide clusters. Furthermore, the $\Delta_R C_P$ values of the different peptides also correlate with their antimicrobial efficiency.

The question now arose, whether titrations performed at 43 °C, where peptide binding induces clusters of liquid-crystalline DPPG, would show the same trends for the different peptides. Fig. 8 shows the measured heat at 43 °C as a function of peptide to lipid ratio.

As already discussed above and shown in Fig. 6 for the binding of c-RRRWFW the appearance of endothermic reaction heats above a certain threshold ratio indicates the formation of liquid-crystalline PG cluster. The formation of PG domains continues upon further peptide addition till the lipid charges are compensated at a peptide/DPPG ratio of 0.33 (not shown). These endothermic peaks are also visible for the binding of the other peptides, but clear differences in the heights of the peaks

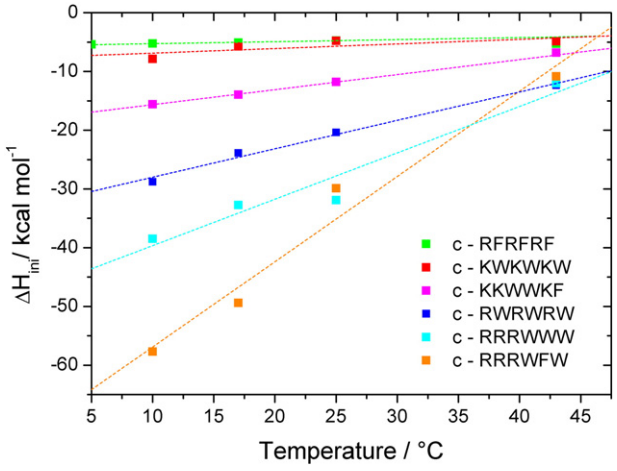

Fig. 7. Averaged first three integrated heats of binding per mol of injected peptide for binding to DPPG/DPPE (1:1) vesicles (2 mM) vs. temperature.

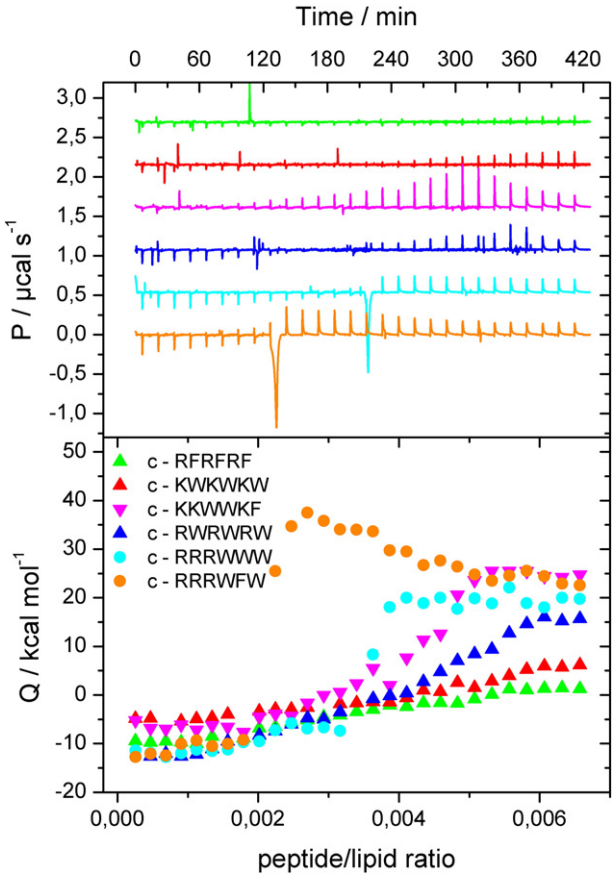

Fig. 8. Top: ITC titration curves (heating power vs. time) of 62 µM cyclic peptide titrated into a 2 mM DPPG/DPPE(1:1) vesicle suspension at 43 °C. Bottom: integrated heats per mol injected peptide vs. peptide-lipid ratio. The high exothermic heats observed for the c-RRRWWW and c-RRRWFW titration are omitted for the sake of clarity.

and also in the threshold value are seen. Peptides which show less clustering efficiency, such as c-RFRFRF and c-KWKWKW display only weak endothermic peaks, whereas these peaks are more pronounced for c-RRRWFW, c-KKWWKF and c-RRRWWW. For c-RRRWFW the reaction heat is between 25 and 34 kcal mol^{-1} of peptide. Assuming a stoichiometry of 3 and complete binding to DPPG in the mixed vesicle this would mean that the endothermic reaction heat is around 8–11 kcal mol^{-1} per DPPG. This corresponds roughly to the heat of transition of pure DPPG at its transition temperature of 41 °C. So the binding data obtained by ITC support the DSC data which show this clustering of almost pure DPPG after peptide binding. For the other peptides the endothermic heats of binding are somewhat lower, meaning that either the stoichiometry is different or the degree of binding is lower. As the sudden increase in reaction enthalpy is less pronounced for the other peptides, weaker binding is more likely. Fig. 9 shows schematically the events that occur after peptide binding at a temperature of 43 °C.

4. Conclusions

We studied the binding of six different cyclic hexapeptides, which were shown before to have antimicrobial activity and to be able to inhibit the growth of *E. coli* and *B. subtilis*, to DPPG and DPPG/DPPE mixed membranes. These peptides are able to cross the outer membrane of Gram-negative bacteria and exert their action on the inner membrane of *E. coli*. [7,40] DSC experiments showed the ability of these trivalent cationic cyclic hexapeptides with different peptide sequences to cluster negatively charged DPPG in a mixed DPPG/DPPE membrane after binding to DPPG. However, the different peptide sequences have an influence on the amount of clustered DPPG at the

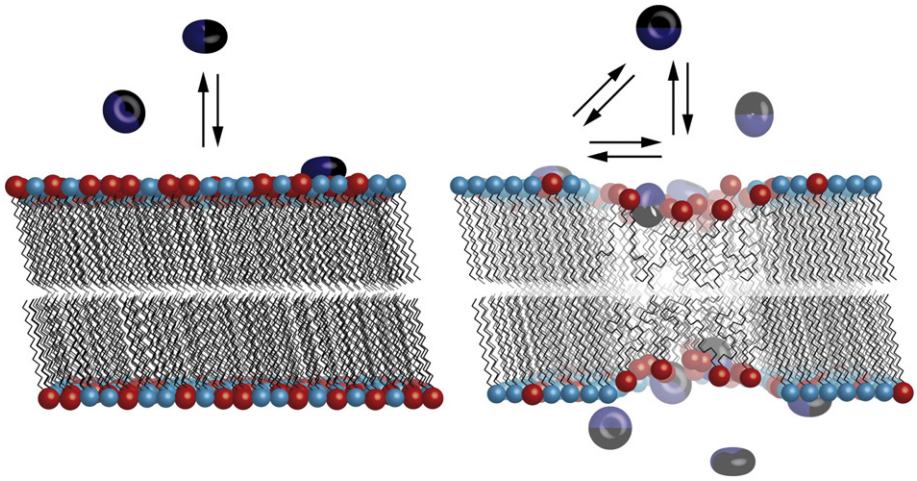

Fig. 9. The scheme displays the interpretation of the ITC data. *Left*: The peptides first bind to the gel phase lipid membrane on the outside of the vesicles. *Right*: Once a certain threshold is reached they can translocate through the membrane and can also bind to the inside. At this threshold ratio they also induce a separated DPPG-peptide cluster in equilibrium with the remaining DPPG/DPPE mixture and free peptide in solution on both sides of the membrane. At 43 °C the induced lipid-peptide cluster undergoes a phase transition that can be detected by endothermic heat of reaction.

same peptide concentration. The ability to induce clustering of DPPG in different amounts correlates with their antimicrobial activity.

Specifically, we could show that the ability to demix DPPG/DPPE membranes depends on the sequence of the peptides and the nature of their amino acids. The amount of clustered lipids increases with increasing amphipathicity of the molecule. The downshift of the phase transition temperature, as shown by DSC, and the high $\Delta_R C_P$ values, determined by ITC, indicate a penetration of the more hydrophobic and amphipathic peptides into the lipid alkyl chain region. The demixing tendency is abetted by the presence of tryptophane and arginine residues. In case of tryptophane this is due to increased hydrophobicity of the side chain favouring interactions with the lipid acyl chains. In the case of arginine it is well known that the charge is more delocalized in the guanidinium moiety compared to lysine where the cationic charge is localized in the NH_3^+ group. Thus the arginine side chain is more hydrophobic than the lysine side chain and can be more easily incorporated into a hydrophobic environment [35]. Also, additional polar interactions via hydrogen bonds are possible. Arginine side chains are localized deeper in the membrane compared to lysine side chains. [31,36] We can therefore conclude that the increased amount of clustered lipid for arginine containing peptides is a result of deeper penetration into the lipid alkyl chain region leading to a frustrated lipid alkyl chain interaction, but also H-bonding of arginine side chains to the headgroups of DPPG cannot be excluded of having effects on the demixing potency. For PG/PE mixtures negative non-ideality parameters are observed indicating the preferential formation of mixed PG/PE pairs. [27] This pair formation is probably driven by headgroup interactions via hydrogen bonds. Arginine side chains might replace hydrogen bonds to the PG headgroup previously donated by the PE headgroups thus creating an additional driving force for PG clustering after peptide binding.

Although we could show that the ability of the peptides to induce PG clustering correlates with their respective antimicrobial potency, the final event leading to the killing of bacteria is still unclear. Lipid demixing could on the one hand be the cause of bacterial cell death, as it can lead to membrane permeabilization and disruption caused by the induction of non-lamellar phases at physiological conditions [8,43] which was shown earlier to occur by the interaction of the cyclic antimicrobial peptide gramicidin S with bacterial membranes [44].

On the other hand changes in the lateral lipid organization could also affect membrane proteins, which are often dependent on specific lipids [45–48], as their functionality relies on the lipid mixture. It has e.g. been shown that the ADP/ATP carrier (AAC) complex in yeast mitochondria dissociates upon depletion of cardiolipin [49,50]. In contrast, depletion of PE stabilizes AAC supercomplexes [51], both resulting in a reduced cytochrome c oxidase activity and a decreased membrane potential. A more detailed study on the antimicrobial activity of the alternating linear hexapeptide RWRWRW-NH_2 on Gram-positive *B. subtilis* has been recently performed by Wenzel et al. [52]. They could show that the cytoplasmic membrane is the target of this trivalent cationic hexapeptide. As a result of RWRWRW-NH_2 addition they observed dissociation of cytochrome c from the outer membrane leaflet resulting in diminished intracellular ATP content and a breakdown of the membrane potential. They could further identify MurG, a membrane protein involved in cell-wall biosynthesis, to dissipate from the cytosolic membrane surface upon addition of RWRWRW-NH_2 and gramicidin S. Wenzel et al. [52] also reported a release of glutamine/glutamate and asparagine/aspartate by *B. subtilis* to the extracellular medium as response of osmotic stress. This is regulated by mechanosensitive channels, indicating that the binding of the short cationic hexapeptide induces lateral pressure. Nevertheless it has also been shown that the mechanosensitive channel MscL senses changes in membrane thickness, due to hydrophobic mismatches [53], which can also occur upon membrane demixing [54].

These studies thus showed that a sequence of biochemical events can follow the first purely physical effect of the bound peptide on the membrane organization. We can therefore speculate that a certain amount of clustered lipid is necessary to lead to membrane protein dissipation and therefore the peptides with the highest impact on lipid demixing would show the highest antimicrobial efficiency. Another possible explanation is that the proteins dissociate from the lipid membrane leaflet as a result of membrane lateral pressure or curvature. It was often suggested that lateral pressure changes and lipid alkyl chain frustration to be the origin of other observations made with antimicrobial peptides, such as the formation of non-lamellar phases, membrane thinning [55], the formation of interdigitated phases [56] and the detergent-like action of AMPs [57]. As we could show here, the antimicrobial efficiency of the peptides correlates with their ability of lipid alkyl chain frustration. We therefore believe this effect is a fundamental first step of antimicrobial action [58].

Summarizing, peptide binding can result in lipid clustering, the induction of curvature or lateral pressure without necessarily leading to membrane permeabilization and cell death. The changes in lipid lateral organization can lead to other effects as described above, namely dissociation of proteins or changes in the function of membrane proteins which require specific lipids. The ability of the cyclic hexapeptides used in this study to induce PG clustering in membranes is remarkably

correlated with their antimicrobial activity. Therefore, it seems possible that this binding induced change in lipid lateral organization is important for their antimicrobial activity. As the demixing effects occur only in membranes composed of PGs and PEs and not in PC/PG membranes, the lipid composition of the membrane plays an important role. The cytoplasmic membranes of bacteria contain besides PG and PE often high amounts of cardiolipins. It would be of great interest whether these effects of demixing also occur in model membranes with more complex mixtures also containing cardiolipins. Indeed, it was shown recently that the binding affinity of c-RRRWFW to POPE/CL mixtures is higher than to POPG/POPE membranes [16]. Additional experiments on structural changes occurring after binding of antimicrobial peptides to membranes with more complex lipid mixtures are therefore necessary to understand the driving forces behind the observed processes and how the interactions between different lipids in the mixed model membrane and with the bound peptides influence the lateral distribution of all components in the plane of the membrane.

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