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Structure and dynamics of the two amphipathic arginine-rich peptides RW9 and RL9 in a lipid environment investigated by solid-state NMR and MD simulations

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ABSTRACT

Cell penetrating peptides (CPPs) are able to cross membranes without using receptors but only little information about the underlying mechanism is available. In this work, we investigate the interaction of the two arginine-rich CPPs RW9 and RL9 with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG), and POPC/POPG membranes with varying POPG content using isothermal titration calorimetry (ITC), solid-state nuclear magnetic resonance (NMR) spectroscopy, and molecular dynamics (MD) simulations. Both peptides were derived from the known CPP penetratin and it was shown previously that RW9 is able to penetrate membranes better than RL9. Overall, the results show that both RW9 and RL9 have a relatively small influence on the membrane. They increase the order of the lipids in the headgroup region and reduce order in the acyl chains indicating that they are located in the lipid/water interface. In addition, the flexibility of the membrane is slightly increased by both peptides but RW9 has a larger influence than RL9. The differences observed in the influences on POPC and POPG as well as MD simulations on the mixed POPC/POPG bilayers of 850 ns length each show that both peptides preferentially associate with and enrich the charged PG lipids almost 2fold in an area of 12 Å around the peptides. As expected, we could not observe any membrane crossing on the simulation time scale of 850 ns but observed that some peptides flipped their orientation during binding to the membrane. Interestingly, all observed flips coincided with structural changes in the peptides indicating that structural changes or flexibility might play a role during the binding of arginine-rich CPPs to membranes.

1. Introduction

Cell penetrating peptides (CPPs) are able to cross cell membranes independently of chiral recognition and for that, they have attracted much attention. They are particularly interesting for medical applications in which a Trojan horse approach is used to deliver cargo over the blood brain barrier or cellular membranes [19]. However, despite their importance little is known about their uptake process. Currently, it is accepted that two pathways coexist. One is based on formation of inverted micelles [16] while the other relies on endocytosis [54,73]. Independently from the internalization mechanism, CPPs interact with and cross the membrane at several stages during their cellular action: first upon their initial contact with the cellular membrane and further after uptake into the cell interior where they may need to enter or exit different intracellular organelles, depending on their target and mode of action. Therefore, a careful investigation on the interaction of these peptides with lipids and their effect on lipid physical chemical properties and organization is necessary.

Herein, we study the interaction of two nine residue arginine-rich peptides (RL9 and RW9) with model lipid membranes of varying composition. RL9 (RRLLRRLRR-NH_2) and RW9 (RRWWRRWRR-NH_2) share a common secondary amphipathic structure and were designed from a structure/function study of penetratin [17]. RW9 was shown to be a potent CPP [6,15,34,71] while RL9 was internalized very poorly although it was able to bind to the cell membrane. Differential scanning calorimetry (DSC) studies showed that both peptides substantially perturbed the main phase transition of negatively charged lipids but did not affect that of zwitterionic ones [71]. Additionally, the interaction of the peptides with negatively charged lipids changed their structure from random to helical [71].

The aim of this study is to understand the basis for the different cellular uptake properties of RW9 and RL9 and to further investigate the interactions between these two peptides and membranes. As for the model membranes employed in this study, two lipid compositions were chosen: POPC as a model of the eukaryotic membrane and POPC/POPG mixtures with varying POPG content to investigate the difference between anionic and zwitterionic lipids [71]. Recent studies showed that

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charged amino acids such as arginine contribute significantly to the binding and potency of CPPs as well as antimicrobial peptides (AMPs) [70]. In contrast molecular dynamics (MD) simulations showed that the interaction between arginines and charged headgroups contribute only little to the binding and translocation of peptides [69]. This contradiction might be solved by the fact that even though the extracellular leaflet of the eukaryotic cell membrane has very few anionic lipids, their clustering into domains upon peptide interaction can potentiate their role as reported for penetratin [36] and a number of other peptides [70]. Moreover, in the possible entrance or exit from the different intracellular organelles, anionic lipids can be encountered. Therefore, aspects concerning peptide insertion into membranes, their orientation, and subsequent lipid reorganization appeared of particular interest to us. For this purpose, several complementary techniques such as isothermal titration calorimetry (ITC), solid-state nuclear magnetic resonance (NMR) spectroscopy and MD simulations were used. We show here that both peptides have a considerably higher affinity for anionic lipids (POPG) than for the zwitterionic lipids (POPC) and lead to POPG clustering. Further, the orientation of the peptides reverses during the binding to the membrane where initially the arginine rich face of the peptides is oriented toward the lipids. Following that, some peptide molecules are able to flip, so that the hydrophobic face of the peptide becomes immersed in the lipid fatty acid chain region. This process appears to be accompanied by peptide structural changes and is irreversible under the time scales accessible to our simulations. Further, we propose that the ability of the peptides to increase the flexibility of the membrane is correlated to their ability to translocate across the membrane.

2. Materials and methods

2.1. Materials

For ITC experiments, the glycerophospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) were obtained as powders from Genzyme (Liestal, Switzerland). Biot(O_2)–Apa–RL9, and Biot(O_2)–Apa–RW9 were obtained from PolyPeptide Laboratories (Strasbourg, France). For the solid-state NMR experiments POPC, POPG, 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC-d_{31}), and 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG-d_{31}) were procured from Avanti Polar Lipids, Inc. (Alabaster, AL). The peptides RW9 and RL9 were produced by standard solid phase peptide synthesis.

2.2. ITC experiments

To prepare large unilamellar vesicles (LUV), appropriate amounts of POPC and/or POPG were combined in chloroform/methanol (2/1 vol/vol) which was then evaporated under a N_2 flow and subsequent placement in a vacuum chamber for 2–3 h to deposit the lipids as a film on the wall of the test tube. Films were then hydrated by addition of the appropriate amount of buffer (10 mM Tris, 100 mM NaCl, 2 mM EDTA, pH 7.5) and vortexed extensively at room temperature. The obtained multilamellar vesicles were submitted to five freeze/thawing cycles and the homogenous lipid suspension was passed 15 times through a mini extruder (Avanti Lipids, Alabaster, AL) equipped with two stacked 100 nm polycarbonate membranes. We used LUVs composed of 100% POPC, 75% POPC 25% POPG, 50% POPC 50% POPG and 100% POPG.

ITC experiments were performed on a TA Instrument (New Castle, DE) nano ITC calorimeter. To avoid air bubbles, peptide and LUVs solutions were degassed under vacuum before use. Titrations were performed by injecting 10 μl aliquots of 100 nm LUVs composed of pure lipid into the calorimeter cell containing the peptide solution with 5 min waiting between injections. The following lipid and peptide concentrations were used: 26 mM lipid and 50 μM peptide for 100% PC LUVs, 13.1 mM lipid and 25 μM peptide for 25% POPG LUVs, 9.8 mM lipid and 50 μM peptide for 50% POPG LUVs, and 6.5 mM lipid and 100 μM for 100% POPG LUVs. The experiments were performed at 35 °C. Data were analyzed and the thermodynamic parameters were derived using the program NanoAnalyze provided by TA Instruments.

2.3. Solid-state NMR

Aliquots of the phospholipids and peptide were co-dissolved in organic solvent, dried under vacuum (10 mbar), and dissolved in cyclohexane. After freezing in liquid nitrogen, the samples were lyophilized under a vacuum of approximately 0.1 mbar. Subsequently, the sample was hydrated to 50 wt.% with deuterium-depleted ^1H_2O, freeze–thawed, stirred, and gently centrifuged for equilibration. The samples were then transferred to 5 mm glass vials and sealed with Parafilm for NMR measurements.

Static ^2H and ^{31}P NMR spectra were acquired with a widebore Bruker DRX 300 NMR spectrometer operating at a resonance frequency of 46.1 MHz for ^2H and 121.6 MHz for ^{31}P. A double-channel solids probe equipped with a 5 mm solenoid coil was used. The ^2H NMR spectra were accumulated with a spectral width of ±250 kHz using quadrature phase detection, a phase-cycled quadrupolar echo sequence [14] with two 6.5 μs 90° pulses separated by a 60 μs delay, and a relaxation delay of 0.5 s while the ^{31}P spectra were acquired with a spectral width of ±250 kHz using a Hahn-echo pulse sequence with a ^{31}P 90° pulse length of 2.5 μs, a Hahn-echo delay of 60 μs and a relaxation delay of 2.5 s. A phase-cycled inversion-recovery quadrupolar echo pulse sequence was used to measure the ^2H NMR relaxation times for the decay of Zeeman order (T_{1Z}; spin–lattice relaxation time). A relaxation delay of 1.5 s was used and all other parameters were the same as for recording the ^2H NMR spectra. An exponential line broadening not exceeding 50 Hz was applied.

For determination of the order parameters first the ^2H NMR powder-type spectra (Fig. S2) were de-Paked using the algorithm of McCabe and Wassall [44]. Order parameter profiles were determined from the observed quadrupolar splittings ($\Delta\nu_Q$) in these spectra according to:

$$\left|\Delta\nu_Q^{(i)}\right| = \frac{3}{2}\chi_Q\left|S_{CD}^{(i)}\right||P_2(\cos\theta)|. \quad (1)$$

Here $\chi_Q = e^2qQ/h$ represents the quadrupolar coupling constant (167 kHz for ^2H in the C–^2H bond [13,59]), θ is the angle between the bilayer director axis and the main external magnetic field, and P_2 is the second Legendre polynomial. For the de-Paked ^2H NMR spectra $\theta = 0°$ and hence $P_2(\cos\theta) = 1$. The segmental order parameter is described by

$$S_{CD}^{(i)} = 1/2\left\langle 3\cos^2\beta_i - 1\right\rangle \quad (2)$$

and depends on the angle β between the C–^2H bond vector and the bilayer director axis where the brackets indicate an ensemble or time average. Further details of the order parameter determination have been described in the literature [31]. The Pake doublets were assigned starting at the terminal methyl group, which exhibits the smallest quadrupolar splitting. The methylene groups were assigned consecutively according to their increasing quadrupolar splittings.

2.4. Molecular dynamics simulations

A total of three all-atom MD simulations was conducted: one reference simulation of a pure membrane of 500 ns length and two simulations of the same membrane in presence of either four RW9 or RL9 peptides of 850 ns length each. Setup of the membrane that consisted of 160 POPC and 40 POPG molecules was conducted following published procedures [35]. Subsequently, four peptides were added on one side of