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Control and role of pH in peptide–lipid interactions in oriented membrane samples

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ABSTRACT

To understand the molecular mechanisms of amphiphilic membrane-active peptides, one needs to study their interactions with lipid bilayers under ambient conditions. However, it is difficult to control the pH of the sample in biophysical experiments that make use of mechanically aligned multilamellar membrane stacks on solid supports. HPLC-purified peptides tend to be acidic and can change the pH in the sample significantly. Here, we have systematically studied the influence of pH on the lipid interactions of the antimicrobial peptide PGLa embedded in oriented DMPC/DMPG bilayers. Using solid-state NMR (^{31}P, ^{2}H, ^{19}F), both the lipid and peptide components were characterized independently, though in the same oriented samples under typical conditions of maximum hydration. The observed changes in lipid polymorphism were supported by DSC on multilamellar liposome suspensions. On this basis, we can present an optimized sample preparation protocol and discuss the challenges of performing solid-state NMR experiments under controlled pH. DMPC/DMPG bilayers show a significant up-field shift and broadening of the main lipid phase transition temperature when lowering the pH from 10.0 to 2.6. Both, strongly acidic and basic pH, cause a significant degree of lipid hydrolysis, which is exacerbated by the presence of PGLa. The characteristic re-alignment of PGLa from a surface-bound to a tilted state is not affected between pH of 7 to 4 in fluid bilayers. On the other hand, in gel-phase bilayers the peptide remains isotropically mobile under acidic conditions, displays various co-existing orientational states at pH 7, and adopts an unknown structural state at basic pH.

1. Introduction

Membrane protein structure and the functional mechanisms of membrane-active peptides can only be fully understood in the presence of a proper lipid bilayer. Therefore – instead of employing detergent micelles, bicelles or organic solvent mixtures – many biophysical studies rely on the use of membrane samples, consisting of multiple stacked bilayers that contain the reconstituted peptide or protein in a liquid crystalline and well hydrated lipid environment [1–3]. Multilamellar vesicles are readily obtained in suspension using aqueous buffers, but a fundamental advantage can be gained by preparing them in a macroscopically oriented manner. This way, anisotropic information about the alignment of a whole protein, of a secondary structure element, or of an individual functional group becomes accessible. The most prominent anisotropic spectroscopic techniques include solid-state NMR (SSNMR) [1,4,5], oriented circular dichroism [6–8], attenuated total reflection infrared (ATR-IR) [9,10], electron paramagnetic resonance (EPR) [9,11,12], as well as scattering methods with X-rays and neutrons [13–16]. In all these methods it is very difficult to control the pH of a sample that has been macroscopically oriented on a solid support, so most of these studies tend to be pursued without pH control. The common use of distilled water together with synthetic peptides that are usually purified under acidic conditions by HPLC implies that the resulting data might suffer from unnaturally acidic conditions. Since the protonation state not only of the peptides/proteins but also of certain lipids can play a critical role in their structure and function, a critical assessment of pH effects and potential pitfalls is timely.

From our experience, SSNMR analysis of membrane-active peptides (MAPs) in oriented lipid bilayers is the most versatile and comprehensive approach to study peptide–lipid interactions. It can provide not

Abbreviations: MAP, membrane active peptides; PC, phosphatidylcholine; PG, phosphatidylglycerol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (Na^{+} salt); PGLa, peptidyl-glycyl-leucine-carboxyamide peptide [GMASKAGAIAGKIAKVALKAL-NH2]; S-state, "surface-bound" state; T-state, "tilted" state; I-state, "inserted" state; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; SSNMR, solid-state nuclear magnetic resonance spectroscopy; ATR-IR, attenuated total reflection infrared spectroscopy; EPR, electron paramagnetic resonance spectroscopy; HPLC, reversed-phase high-performance liquid chromatography; DSC, differential scanning calorimetry; Lα, fluid lamellar phase; L_{β}, lamellar gel phase; P_{β}, rippled gel phase; T_m, main transition temperature (P_{β}-L_{α}); T_p, pre-transition temperature (L_{β}-P_{β}); P/L, peptide-to-lipid (molar) ratio

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only a structural description (conformation, membrane alignment) [1,4,17,18] and dynamical information (molecular wobble, lateral diffusion, aggregation) [19–27] about the MAP in its functionally relevant membrane-bound state, but it can also monitor the response of the phospholipid matrix (lipid disorder, morphological transitions) [28–31]. The samples typically consist of stacks of several 1000 hydrated multibilayers that are spontaneously aligned on an inert solid support, like glass, quartz or silicon. They are very suitable as proper membrane models, because the lipid composition can be freely chosen [32–34], the peptide concentration can be varied over several orders of magnitude [17], the bilayers can be observed in their desired phase state, and it is easy to control the membrane charge [34,35], bilayer thickness [34,36], fluidity, spontaneous curvature [32,33]. Several reports on SSNMR applications have been concerned with optimizing the sample preparation, e.g. to achieve perfect alignment, but they were mainly focussed on adjusting the solvent composition for co-solubilizing peptide and lipids [38–41]. A commonly underestimated aspect, however, is the fact that many studies are carried out with synthetic peptides rather than recombinantly produced material. Synthetic peptides are routinely purified by commercial suppliers and in academic labs with RP-HPLC, which usually employs acidic eluents. If no post-purification treatment (e.g. neutralisation) is performed, any HPLC purified peptide carries with itself a stoichiometric excess of acid (commonly trifluoroacetic acid) [1,42–44]. When no sufficient buffer capacity is provided in the sample, this acid introduced by the peptide may significantly influence the system under study. A resulting effect can be, for instance, acid hydrolysis of the phospholipid molecules, leading to the generation of lyso-lipids, which will affect the membrane composition and properties [45,46]. Lipid hydrolysis as a factor modulating the behaviour of magnetically oriented "bicelles" has been intensively studied, and ether-linked phospholipids with reduced risk of hydrolysis are often used to circumvent this problem [47–49]. However, even this lipid choice is not a perfect solution, because the structure and alignment of MAPs in ether- vs. ester-linked phospholipid bilayers has been reported to differ [50,51].

Peptide-induced acidification or "spontaneous" hydrolysis in the peptide-containing samples is a practical problem that all experimental NMR spectroscopists must be aware of when using solid-supported oriented samples. In many cases the consequences are ignored, or precautions simply involve an acquisition of ^{31}P NMR spectra before and after the actual SSNMR measurement in order to assess the "damage" and to be alerted before interpreting the data. To the best of our knowledge, the problem of active pH control in oriented samples has not been explicitly discussed in the literature. Furthermore, it is well documented that lipid polymorphism depends not only on temperature, ionic strength and composition of the environment, but also on pH [52–55]. Since various ionizable groups are present in lipid membranes (Table 1), the pH of a sample can directly influence the membrane charge, spontaneous curvature and lateral pressure profile by protonation/deprotonation of the lipid headgroups. The protonation state of lipids and peptides can be further affected by the presence of anionic lipids, as they tend to lower the pH at the bilayer surface compared to the bulk [56].

Also the peptidic component in a membrane sample can be affected by pH. The HPLC derived acidity is not strong enough to cause hydrolysis of the amide backbone, but the ionization state of side chains can be easily affected, and thereby the peptide conformation and its interactions with the bilayer [56–59]. A prominent example is the pH-dependent re-orientation of amphiphilic helices triggered by the protonation/deprotonation of histidines [60]. Such pH-induced structural transitions have been explored to trigger the activity of viral fusion peptides and of ion channels, and in the design of pH-responsive materials [61–67]. Another pH-sensitive group on peptides is the N-terminus, which has a pKa value close to 7 and may accordingly interact in different ways with the amphiphilic bilayer.

Table 1
Ionizable groups in common phospholipids and their reported pKa values [54].

Phospholipid:	Ionizable group:	pKa
Phosphatidylcholines	phosphate	<2, 2–3
Phosphatidylglycerols	phosphate	3–3.5
Phosphatidyletanolamines	phosphate	<2, 2–3
	amine	8–11.25
Phosphatidylserines	phosphate	1.2
	carboxyl	3.6–5.5
	amine	9.8–11.5

Here, we address the fundamental question as to what extent typical SSNMR experiments can be influenced by pH over a broad range. Namely, whether/which pH would induce changes in the bilayer properties, and whether/how would the alignment of an α-helical peptide (without histidines) change upon varying the pH? To address these issues in a suitable test system, we chose a representative mixture of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in a molar ratio of 3:1. Such anionic mixtures are often used as bacterial membrane mimics [1,4,34] and are therefore relevant for studies of antimicrobial MAPs. The polymorphism of both lipids has been well characterized, the two lipid species are almost ideally miscible [68], and their phase transitions occur in a convenient temperature regime. DMPC/DMPG mixtures are widely used to examine changes in other experimental conditions [69–71], both alone as well as in the presence of peptides or proteins [72,73]. This particular mixture of 3:1 has also been used extensively before in our earlier SSNMR studies of the present test peptide PGLa [17,34,35,37].

PGLa was selected as an exemplary peptide, being a typical cationic antimicrobial membrane-active peptide with 21 amino acids (GMASKAGAIAGKIAKVALKAL-NH_2) [74]. The peptide is unstructured in aqueous solution but adopts an amphiphilic α-helical structure in membrane-mimicking environments [75]. PGLa has been comprehensively characterized by SSNMR, revealing several different orientational states depending on the conditions. The helix undergoes a concentration dependent re-alignment in DMPC/DMPG bilayers at room temperature [17]. At low peptide-to-lipid ratios (e.g. 1:200) PGLa lies flat on the membrane in a surface-bound "S-state", while at higher concentration ($P/L \geq 1:50$) it adopts a tilted "T-state" that has been attributed to the formation of antiparallel dimers [17,76]. When mixed with its synergistic partner magainin 2, hetero-oligomers are formed in which the PGLa helix becomes aligned nearly parallel to the membrane normal [33]. This fully inserted "I-state" suggests that transmembrane pores have been formed, which can explain the antimicrobial mechanism. The I-state was also observed for PGLa alone at high peptide/lipid ratios (e.g. 1:50) in metastable gel-phase bilayers [37]. Changes between the three states (S, T, I) can be monitored by solid-state ^{19}F NMR, e.g. by observing a selectively ^{19}F-labelled peptide carrying a CF_3-containing amino acid, e.g. in the place of Ile13 [34]. This particular PGLa analogue is a good candidate to examine the influence of pH on the re-alignment behaviour of this well-known MAP.

Besides the ^{19}F NMR experiments on PGLa, we also used ^{2}H NMR and ^{31}P NMR to monitor the pH-dependent response of the DMPC/DMPG matrix. In addition, differential scanning calorimetry (DSC) measurements on isotropic lipid dispersions with different pH values were performed with multilamellar liposomes. As there is no ambiguity in pH or ionic strength in these samples, they provide an independent reference for the changes in lipid polymorphism and peptide–lipid interactions that are observed in the oriented NMR samples.

2. Materials and methods

2.1. Peptide, lipids, buffers

PGLa was synthesized using standard Fmoc-protocols, either as the native sequence or with a single CF_3-Bpg (2-amino-2-[3-(trifluoromethyl)bicyclo[1.1.1]pent-1-yl]ethanoic acid, obtained