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Comparison of lipid-dependent bilayer insertion of pHLIP and its P20G variant

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ABSTRACT

The ability of the pH-Low Insertion Peptide (pHLIP) to insert into lipid membranes in a transbilayer conformation makes it an important tool for targeting acidic diseased tissues. pHLIP can also serve as a model template for thermodynamic studies of membrane insertion. We use intrinsic fluorescence and circular dichroism spectroscopy to examine the effect of replacing pHLIP's central proline on the pH-triggered lipid-dependent conformational switching of the peptide. We find that the P20G variant (pHLIP-P20G) has a higher helical propensity than the native pHLIP (pHLIP-WT), in both water:organic solvent mixtures and in the presence of lipid bilayers. Spectral shifts of tryptophan fluorescence reveal that with both pHLIP-WT and pHLIP-P20G, the deeply penetrating interfacial form (traditionally called State II) is populated only in pure phosphocholine bilayers. The presence of either anionic lipids or phosphatidylethanolamine leads to a much shallower penetration of the peptide (referred to here as State II^S, for "shallow"). This novel state can be differentiated from soluble state by a reduction in accessibility of tryptophans to acrylamide and by FRET to vesicles doped with Dansyl-PE, but not by a spectral shift in fluorescence emission. FRET experiments indicate free energies for interfacial partitioning range from 6.2 to 6.8 kcal/mol and are marginally more favorable for pHLIP-P20G. The effective pKa for the insertion of both peptides depends on the lipid composition, but is always higher for pHLIP-P20G than for pHLIP-WT by approximately one pH unit, which corresponds to a difference of 1.3 kcal/mol in free energy of protonation favoring insertion of pHLIP-P20G.

1. Introduction

The discovery of pHLIP (pH-Low Insertion Peptide) is a compelling example of how a basic-science study can be translated into a very useful and practical biomedical tool. While originally designed from the sequence of the C-helix of bacteriorhodopsin to study membrane protein folding [1], pHLIP is currently and most often used for imaging tumors and other acidic diseased tissues [2–9], as well as for targeted drug delivery [10–13]. The underlying mechanism of pHLIP's pH-dependent action is associated with the protonation of Asp residues, allowing for membrane insertion at acidic pH [14–16]. Many other mechanistic and thermodynamic aspects of membrane interactions of pHLIP, however, remain the subjects of active studies.

Traditionally, membrane interactions of pHLIP are described in the context of the following three states, which were originally identified for partitioning into phosphatidylcholine (PC) bilayers [17]: unfolded and soluble state in water at neutral pH (State I), unfolded interfacial state at neutral pH (State II), and a transmembrane α-helical state at low pH (State III). Each of the states has a defined spectroscopic signature associated with the amount of secondary structure (measured by circular dichroism) and membrane penetration (blue shift of Trp fluorescence spectrum relative to that of the solution State I). In our previous study we challenged this view for bilayers of mixed lipid composition by demonstrating that the spectroscopic signature of the interfacial State II is only observed in pure PC bilayers [18].

A surprising feature of pHLIP is the apparent lack of folding upon interfacial penetration, which sets it apart from numerous membrane active peptides that undergo a partitioning-folding coupling [19–21]. To examine the effects of helical propensity on membrane interactions of pHLIP, we examine here the folding and membrane interactions of the native peptide (referred to here as the wild type, pHLIP-WT) and those of the P20G variant (pHLIP-P20G), lacking the helix-breaking proline in the middle of the hydrophobic stretch. Both pHLIP-WT and pHLIP-P20G have been previously used by Engelman and co-workers

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[15] to investigate the effect of acyl chain-length on membrane insertion in the context of phosphatidylcholine membranes. They have demonstrated that both membrane thickness and fluidity modulate pHLIP membrane insertion and that Pro20-to-Gly replacement resulted in a more favorable membrane insertion. By examining the pH range from 4.5 to 8, the authors concluded that the insertion pK_a for pHLIP-P20G is less acidic than that of the pHLIP-WT, with the difference ranging from 0.1 to 1.0 pH units, for 22 and 14 carbon lipids, respectively. Here, we reexamine the pH-titration of both peptides into both pure POPC bilayers and bilayers containing 25% POPE or 25% POPS in POPC matrix. We find that a wider range of pH (4.5–10) is necessary to accurately characterize the insertion and that the difference for the insertion of the two peptides may be larger than reported. Our results indicate that, in all three lipid compositions, the difference is approximately 1 unit of pH and that, consistent with our previous report [18], lipid headgroups modulate the insertion of pHLIP. We introduce a revised version of the insertion scheme, which contains an alternative interfacial State II^S, to indicate a more "shallow" bilayer penetration as compared to traditional interfacial State II.

2. Materials and methods

2.1. Materials and lipid abbreviations

N-Hydroxybenzotriazole (HOBt), *o*-benzotriazol-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and all *N*-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids were purchased from GL Biochem Ltd. H-Rink Amide-ChemMatrix solid support resin was purchased from PCAS BioMatrix Inc. Diisopropylehtylamine (DIEA), piperazine, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM) and trifluoroacetic acid (TFA) were from Thermo Fisher Scientific Inc. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospholcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS).

2.2. Peptide synthesis

pHLIP-WT (H_2N-AAEQNPIYWARYADWLFTTPLLLLDLALLVDADEGG-$CONH_2$) and pHLIP-P20G (H_2N-AAEQNPIYWARYADWLFTTGLLLLDLAL LVDADEGG-$CONH_2$) were prepared by Fmoc solid-phase synthesis as described previously [10–13], using H-Rink Amide-ChemMatrix resin affording an amidated C-terminus and purified via reverse-phase high performance liquid chromatography (RP-HPLC) (Phenomenex Luna prep 10 μ 250 × 21.20 mm C8; flow rate 10 mL/min; phase A: water 0.1% trifluoroacetic acid (TFA); phase B: acetonitrile 0.1% TFA; gradient 60 min from 95/5 A/B to 0/pure A/B). The purity of the peptides was determined by RP-HPLC (Agilent Zorbax Eclipse 5 μm 4.6 × 50 mm XDB-C8; flow rate 1 mL/min; phase A: water 0.01% TFA; phase B: acetonitrile 0.01% TFA; gradient 45 min from 95/5 A/B to 0/pure A/B) and their identity was confirmed via matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

2.3. Preparation of lipid vesicles

Large Unilamellar Vesicles (LUV) were prepared by drying the required volume of chloroform lipid stocks under a nitrogen stream before overnight drying using high vacuum. Dried lipid films were resuspended in 50 mM phosphate buffer (pH 8) to a final concentration of 20 mM and vortexed. For FRET measurements, 2% of Dansyl-PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) was incorporated into lipid mixture. LUV were formed by extrusion using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) through nucleopore polycarbonate membranes of 0.1 μm pore size (Whatman, Philadelphia, PA) [22,23]. LUV stocks were prepared in 50 mM phosphate buffer, pH 8, and stored at − 4 °C. No lipid degradation was detected using thin layer chromatography.

2.4. Sample preparation

2 μM of pHLIP were mixed and incubated with LUV stock in 10 mM phosphate, pH 8 or 10 mM borate buffer, pH 10, resulting in molar lipid to peptide ratios of at least 500:1 (1–2 mM LUV). Membrane insertion of pHLIP was initiated by addition of the appropriate aliquots of 0.5 M acetate buffer. To avoid potential lipid degradation, the lipid-containing samples were never kept at pH 10 longer than 20 min.

2.5. Fluorescence measurements

Fluorescence emission was measured using a SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ) equipped with double-grating excitation and emission monochromators. The measurements were made in a 2 × 10 mm cuvette oriented perpendicular to the excitation beam and maintained at 25 °C using a Peltier device from Quantum Northwest (Spokane, WA). For tryptophan fluorescence measurements, the excitation wavelength was 285 nm and the slits were 4 and 6 nm for emission and excitation, respectively. For acrylamide quenching, 295 nm excitation was used to reduce inner filter effects and 3 nm slits. The appropriate background spectra were subtracted in all cases. Spectral analysis was carried out using Origin 8.5 (OriginLab, MA).

2.6. Membrane partitioning

Membrane partitioning was measured by fluorescence titration of peptides with LUV containing 2% Dansyl-PE as acceptors. Interfacial partitioning is indicated by a decrease in the intensity of the donor Trp peak as it associates with the interface as a function of LUV concentration (Fig. S4). Results are plotted as the relative decrease in Trp intensity, I, versus lipid concentration, [L], and fitted as described previously using the following equation [24]:

$$I = 1 + (I_\infty - 1)\left(\frac{K_x \cdot [L]}{[W] + (K_x \cdot [L])}\right) \quad (1)$$

where I_∞ denotes the relative intensity at infinite lipid saturation, [W] is the concentration of water (55.3 M) and K_x represents the mole fraction partitioning coefficient [25]:

$$K_x = \frac{[P_{bil}]/[L]}{[P_{water}]/[W]} \quad (2)$$

where $[P_{bil}]$ and $[P_{water}]$ corresponds to the bulk concentrations of peptide in the bilayer and in water respectively. The calculated partitioning constant (K_x) was used to determine the free energy of interfacial partitioning (ΔG_{IF}) using the following formula:

$$\Delta G_{IF} = -RT \cdot \ln(K_x) \quad (3)$$

where R is the gas constant (1.985×10^{-3} kcal K^{-1} mol^{-1}) and T is the experimental temperature in Kelvin (298 K). Errors in the fits were determined by support plane analysis [26] and are indicated in brackets (Figs. S5–6).

2.7. Analysis of the pH-dependent membrane insertion

The spectral data were fitted by applying nonlinear least-square analysis with the following equation [27]:

$$\lambda = \frac{\lambda_N + \lambda_L \times 10^{m(pK_a - pH)}}{1 + 10^{m(pK_a - pH)}} \quad (4)$$

where λ_N and λ_L are the limiting values of fluorescence maximum at neutral and low pH, respectively; pK_a is an apparent constant and m is a transition slope. Errors in the fits were determined by support plane analysis and are indicated in brackets (Figs. S7–8). The protonation-