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Tuning the Insertion Properties of pHLIP

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Abstract

The pH (low) insertion peptide (pHLIP) has exceptional characteristics: at neutral pH it is an unstructured monomer in solution or when bound to lipid bilayer surfaces, and it inserts across a lipid bilayer as a monomeric alpha-helix at acidic pH. The peptide targets acidic tissues *in vivo*, and may be useful in cancer biology for delivery of imaging or therapeutic molecules to acidic tumors. To find ways to vary its useful properties, we have designed and analyzed pHLIP sequence variants. We find that each of the Asp residues in the transmembrane segment is critical for solubility and pH-dependent membrane insertion of the peptide. Changing both of the Asp residues in the transmembrane segment to Glu, inserting an additional Asp into the transmembrane segment, or replacing either of the Asp residues with Ala leads to aggregation and/or loss of pH-dependent membrane insertion of the peptide. However, variants with either of the Asp residues changed to Glu remained soluble in an aqueous environment and inserted into the membrane at acidic pH with a higher pK_{app} of membrane insertion. Variation of the pH at which insertion occurs may broaden the potential uses of the pHLIP technology by allowing targeting of tissues with different acidity levels.

Keywords

pHLIP; Transmembrane helix; membrane insertion; helix formation

INTRODUCTION

Analysis of a 36-residue polypeptide containing the sequence of the bacteriorhodopsin C-helix revealed a peptide with exceptional properties (1). The peptide is soluble in aqueous solution and associates with lipid bilayers as an unstructured monomer at neutral pH, and inserts across a bilayer or membrane as an alpha-helix at acidic pH. Thus the peptide has been named pH (low) insertion peptide (pHLIP) (2). pHLIP's exceptional properties are being applied in cancer biology, where acidosis is a known feature of solid tumors. Tissue acidity is also found in other pathological conditions, such as inflammation sites and focal lesions caused by infection, ischemia and stroke. pHLIP insertion into cell membranes at low pH (pH below neutral) has been shown to localize tumors and inflammation sites in mice and rats (3,4). The variations of acidity in different tumors or different diseased tissues are not well understood, but it is likely that significant differences exist, and variations among tumors are suggested by some studies (REFERECES HERE). Thus, an ability to tune the pH of insertion of pHLIP could be an advantage in differentially imaging and treating tissues of differing acidity.

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In addition to selective targeting of acidic solid tumors *in vivo* pHLIP has been used to translocate cargo molecules into the cytoplasms of cultured cancer cells (4). This phenomenon may allow the use of the peptide to deliver relatively polar therapeutic molecules to cells in acidic tissues. Further, the peptide might be exploited to study the process of spontaneous peptide folding and insertion into lipid bilayers. Amphipathic peptides have been used to understand the kinetics, thermodynamics or structures of membrane proteins (5-10). It is well established that the folding of a single helix can initiate the assembly of more complex membrane proteins (8,11-17), so the study of pHLIP insertion can extend such understanding, particularly since it remains a monomer in all stages.

It is known that pHLIP peptide is monomeric at concentrations up to 7 μM both in aqueous solution and in lipid bilayers (2). In the formation of a transmembrane helix at low pH, the polar C-terminus is reversibly inserted into the cytoplasm while the N-terminus remains on the outside (2). Most recently it has been shown that the peptide binding interaction with the lipid surface is strong, and it has been proposed that the peptide associates with the surfaces of membranes *in vivo*, such as the blood cell membranes, until it finds itself in an acidic environment and only then inserts across the membranes of cells in that environment(18). It is also important to note that membrane integrity is preserved at all stages of peptide interaction (19). The peptide loses its pH dependent insertion properties when the two Asp residues from the transmembrane segment are replaced with either two Asn or Lys residues (4). This finding points to the involvement of these two Asp residues, or at least one of them, in the pH dependent membrane insertion. Further, changes in the peptide by neutralizing the C-terminus lead to helix formation in solution and loss of insertion properties (19). We are working to further understand the roles of different sequence features in solubility and insertion, and in this study we report further evidence for the role of the two Asp residues, and the discovery that the pK of insertion can be varied while preserving useful properties of the peptide. Such variation informs us further about membrane insertion and may broaden the applicability or specificity of the pHLIP technology in the clinic.

METHODS

Peptide preparation

Variants of pHLIP were made by solid-phase peptide synthesis using standard 9-fluorenylmethyloxycarbonyl chemistry and purified by reverse phase chromatography (on a C18 column) at the W.M. Keck Foundation Biotechnology Resource at Yale University (New Haven, CT). The lyophilized peptide was dissolved in a solution containing 6 M urea and dialyzed against 5 changes of 1 L of phosphate buffer (50 mM Na_2HPO_4, 10 mM NaCl, pH 8) each. The concentration of the peptide was determined by absorbance using ε_{280} = 13, 940 M^{-1} cm^{-1}.

Liposome preparation

Large unilamellar vesicles were prepared by extrusion (20). POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) (Avanti Polar Lipids, Inc., Alabaster, AL) lipids in chloroform were de-solvated with N_2 and dried under vacuum at room temperature overnight. The phospholipid film was rehydrated in 50 mM Na_2HPO_4, 10 mM NaCl, pH 8, freeze-thawed 7 times, and passed through the extruder (200 nm membrane) 15 times.

Tryptophan fluorescence and CD measurements

Tryptophan fluorescence and CD measurements were carried out on an ISS spectrofluorometer and a Jasco 810 spectropolarimeter, respectively, under regulated temperature control. Tryptophan fluorescence spectra were recorded from 310 nm to 450 nm at an excitation wavelength of 295 nm, with the spectral width of the excitation and emission slits set at 2-4

nm and 2 nm, respectively. Trp fluorescence and CD spectra of the peptides at 7 μM, unless otherwise mentioned, were measured in phosphate buffer of different pHs in the absence or presence of POPC liposomes. To avoid aggregation of the peptide at low pH, the lipid vesicles were added first and the peptide was added last, although we have found that some versions of the peptide can still insert when lipid vesicles are added to aggregated peptide in a buffer at pH 4 (data not shown). The pH was adjusted by addition of microdrops of diluted NaOH or HCl.

RESULTS AND DISCUSSION

The objective of our study was to use sequence variation of pHLIP to better document key properties of the peptide, and to identify peptides with useful membrane targeting variations. We demonstrated earlier that the simultaneous replacement of two key Asp residues located in the transmembrane part of pHLIP by Lys or Asn led to the loss of pH-sensitive insertion into membranes of liposomes, red blood cells, and cancer cells, as well as to the loss of specific accumulation in tumors *in vivo* (4). Individual variations of the Asps were not investigated, nor were more conservative substitutions. To further clarify the role of the two individual Asp residues, we replaced each of them with Ala (the D14A and D25A variants). Additionally, we studied the possibility of tuning the pKa of pHLIP insertion across a membrane, and/or enhancing the cooperativity of the transition. Thus, several pHLIP variants were designed and their interactions with lipid bilayer of POPC vesicles were studied by fluorescence and CD spectroscopy.

Design of pHLIP variants

We guided the design of pHLIP sequence variations using the apparent free energy difference (ΔG_{app}) for membrane insertion. The calculations were performed using the algorithm developed by Gunnar von Heijne and colleagues (http://www.cbr.su.se/DGpred/index.php?p=TMpred), which is based on the experimental analysis of TM helix insertion into a membrane by the Sec61 translocon (21,22). A negative value of ΔG_{app} indicates that the sequence would be likely to adopt a stable transmembrane helix (TM) configuration. The apparent free energy difference depends on the hydrophobic moment ($\Delta G_{hyd\ mom}$), the length of the helix (ΔG_{length}) and the position of polar amino acids within the transmembrane segment (ΔG^{aa}_{app}). Using this tool we designed the peptides sequences presented in table 1, with the corresponding predicted ΔG_{app} values shown in table 2. All predicted values of the apparent free energy difference are calculated based on the assumption of neutral pH, where Asp residues carry negative charges, therefore, transferring them into the hydrophobic core of a membrane accounts for the large energetic penalty and calculations provide positive values of ΔG_{app}. As expected, our experimental data suggest that pHLIP does not insert across a membrane at neutral pH, while decreasing the pH leads to protonation of the Asp residues and increase peptide hydrophobicity, shifting the equilibrium toward the inserted TM configuration of the peptide. Our overall goal in designing new sequences was to preserve the major properties of WT-pHLIP, particularly monomeric solubility in aqueous solution and an ability to insert across a membrane in a pH-dependent manner. Therefore, we selected variants with similar values of ΔG_{app} to that of WT-pHLIP.

Three states of WT-pHLIP

The fluorescence emission of tryptophan residues and circular dichroism signals of WT-pHLIP are distinct in each of the three states (Fig. 1) and were used to follow membrane surface adsorption and insertion of the peptide (and its variants) across a bilayer (Figure 1) (23). WT-pHLIP in solution at low concentrations is unfolded and monomeric, and the tryptophan residues are exposed to the polar environment, resulting in an emission spectrum with a maximum around 349 nm (Table 3), which is characteristic for class III tryptophan residues

fully exposed to water (24). In the presence of POPC vesicles, the tryptophan fluorescence of WT-pHLIP is blue shifted and the intensity is increased, while the peptide remains unstructured as observed from the CD spectrum. When the pH is decreased to 4, tryptophan fluorescence shifts further (since tryptophan residues are moved into the hydrophobic core of the bilayer), the intensity increases (due to lower water quenching of tryptophan emission), and the peptide becomes alpha helical. All changes of fluorescence and CD point to the peptide being associated in an unstructured fashion with the lipid surface at pH 8 and inserted into the lipid bilayer as an alpha helix at pH 4, and the transmembrane orientation of WT-pHLIP was established earlier by FTIR analysis (14).

Ala-pHLIP variants

Titration of the two Asp carboxyl groups to make them more hydrophobic is our working model for the trigger of insertion and TM formation. To further test the role of each Asp residue in the mechanism of pHLIP insertion at low pH, we replaced each of them by turn with Ala. Calculations gave negative values of ΔG_{app} for both Ala variants (D14A and D25A), indicating that the peptides might adopt transbilayer helical conformations at neutral pH, and we observed the presence of some elements of secondary structure for both peptides in presence of liposomes (Figure 3). The observed helical content increases with a drop of pH, most probably due to protonation of the single remaining Asp residue. It is interesting to note that the prediction indicates that D25A has a higher probability to form a TM helix at neutral pH than the D14A variant. Our fluorescence and CD data show that D25A is partly helical and, probably, more aggregated in solution in the absence of lipids, since the position of the fluorescence maximum is blue shifted (339 nm in contrast to 342 nm for D14A and 349 nm for WT-pHLIP) (Table 3). Also, the addition of lipids at pH 8.0 leads to a further blue shift and enhancement of the quantum yield, while a drop of pH does not lead to any other changes of the fluorescence signal of the D25A variant. At the same time, we observed a progressive blue shift and increase in fluorescence intensity for D14A as a result of the addition of lipids and a drop of pH. The results indicate that both Asp's at position 14 and 25 are vital for maintaining the solubility of the peptide and its insertion properties. These findings are in agreement with our results indicating that 1.5-1.8 H^+ are involved in the protonation reaction (18).

3D-Asp-pHLIP variant

We thought that it would be desirable, if possible, to increase the cooperativity of insertion in order to target a narrower pH range. For this purpose, we designed a peptide with an additional Asp residue positioned between two inherent Asp residues, the “3D” peptide. The 3D peptide was designed to have values of ΔG_{app} comparable to WT-pHLIP (Table 2). Replacement of 19Thr with Leu and insertion of another Leu at position 28 was required to make up for the hydrophilicity of the added Asp and to ensure that the peptide would be hydrophobic enough to insert into a membrane (Table 1). Disappointingly, the CD spectrum of the 3D peptide in solution showed significant helical character even at a concentration of 3 μM (data not shown), and the emission of tryptophan residues was blue shifted (344 nm), pointing to an aggregated state of the peptide in solution even at low concentrations. No further increase in tryptophan fluorescence intensity is observed for the 3D peptide in the presence of POPC vesicles at either pH 8 or pH 4, although a blue shift from 344 nm to 336 nm is apparent with the addition of POPC at pH 8 (Figure 3A and Table 3). CD spectra of the peptide in the presence of liposomes indicate helix formation at pH 8, where WT pHLIP has no helical structure (Figure 3B).

We conclude that the peptide lost key pHLIP properties. While the difference in hydrophobicity is not significant, a notable difference of the calculated parameters for the 3D and WT-pHLIP is in values of the hydrophobic moment (0.64 for 3D vs 0.39 for WT), indicating that a 3D peptide helix would have more amphipathic character than WT, and perhaps suggesting a

design refinement. It may be that the 3D peptide does not insert into a lipid bilayer at neutral pH, but adopts a surface helical configuration as many amphipathic peptides do.

Glu-pHLIP variants

With the aim of differentially targeting tissue regions of various acidity, we explored the possibility of tuning the pH at which pHLIP inserts across a membrane. We studied peptides where one or the other of the TM Asp residues is replaced with Glu (D14E and D25E). We expected that these changes would have only modest effects on hydrophbicity, and calculation revealed little alteration in the parameters of $\Delta G^{aa(i)}_{app}$, hydrophobic moment and peptide length (Table 2). Thus, the apparent free energy differences for both Glu variants are close to the WT-pHLIP, and fluorescence and CD spectra of each variant in the absence and presence of lipids are similar to the WT-pHLIP. In the presence of lipids at pH 8, the tryptophan fluorescence spectrum is shifted from 349 nm to 341-342 nm and its intensity increases, and at pH 4 the spectrum is shifted further to 336 nm with an additional increase of fluorescence intensity (Fig. 3A and Table 3). The peptides remain unstructured in the presence of liposomes at pH 8 and become helical at pH 4, as observed from CD signals (Fig. 3B). These findings are consistent with peptide binding to the lipid surface at pH 8 and TM formation across a lipid bilayer at pH 4, like WT-pHLIP.

Encouraged by the data obtained with the D14E and D25E variants, we studied the double mutant with both Asp residues in the transmembrane domain replaced by Glu, the D14/25E peptide. A negligible difference of calculated parameters was obtained for the D14/25E variant in comparison with WT-pHLIP (Table 2). However, the peptide has very different behavior in solution. The fluorescence spectrum of the D14/25E variant is 7 nm shifted to shorter wavelengths (342 nm vs. 349 nm of WT-pHLIP) and the helical structure is observed at very low peptide concentrations. Also noticeable is the enhanced quantum yield of the peptide in solution (4 times higher than for WT-pHLIP and the D14E and D25E variants), which could be from aggregation or altered peptide configuration such that Trp residues are shielded from water molecules or other peptide groups that might quench tryptophan emission. No changes are observed in tryptophan fluorescence when POPC vesicles are added at pH 8. Although a small blue shift and increase in fluorescence intensity was observed at pH 4 in the presence of liposomes, it is comparable to the fluorescence spectrum of the peptide in solution at pH 4, when the peptide is aggregated (Figure 3A). The CD spectrum for the peptide shows a shift in minima indicative of increased helicity when the pH is decreased from pH 8 to pH 4 (Figure 3B).

Our results show that pHLIP configuration in solution is easily perturbed by seemingly small sequence changes, which lead to the formation of some elements of helical structure and a loss of pH-dependent membrane-insertion properties. Therefore, the design of new sequences should be based not only on the calculation of the stability of TM helices, but also a more challenging evaluation of the propensity to form helical structure in solution and to aggregate. There is a known importance of the position of various amino acids in the alpha-helices of globular proteins, for example it is known that Ala has the highest tendency to be found in the middle of an alpha-helix, and, unexpectedly, that Glu has a much higher tendency than Asp to form a helix in aqueous solution (25). This might explain why helix formation in solution was found when the two Asp residues were simultaneously replaced with Glu residues.

Tuning pK_a of insertion

Several pHLIP variants have been analyzed in this and other studies, and almost all of them lack the key pHLIP property of pH-dependent membrane insertion (4,19). Only two variants, the D14E and D25E peptides, remain soluble and monomeric in solution and insert across a lipid bilayer in a pH-dependent fashion like WT-pHLIP. We evaluated the pK_a values of

insertion of each variant in comparison with WT-pHLIP. The position of the fluorescence maximum of each peptide in the presence of POPC vesicles was measured as a function of pH. The data were fitted using a nonlinear regression model, giving a pK_a of 5.96±0.05 for the WT-pHLIP (Table 4), which is consistent with the previous reports (14). Importantly, the titration curves for the variants were found to be shifted to higher pH, indicating that each peptide requires a less acidic environment for membrane insertion than WT-pHLIP (Fig. 4).

Conclusion

We have tested sequence variants of pHLIP to learn which features are variable within the requirement that the pHLIP properties of solubility and pH-dependent insertion across a membrane are preserved. We showed that the two TM Asp residues are each important, and we found that most sequence variations compromise one or more of the key pHLIP properties. It is clear that the design of new sequences should be based not only on the ability to form a TM helix, but also on minimizing the propensity for aggregation and helix formation in aqueous solution. Of the variant sequences studied, only single Glu substitutions of each of the two Asp positions in the transmembrane helix preserved pHLIP properties, while resulting in an increase of the pKa of insertion by half a pH unit. As a result, we have found two new pHLIP variants, which might be used to selectively target tissues having different degrees of acidity. These variants will now be tested for translocation of molecules across membranes and for targeting of acidic tissues, including tumors, *in vivo*.

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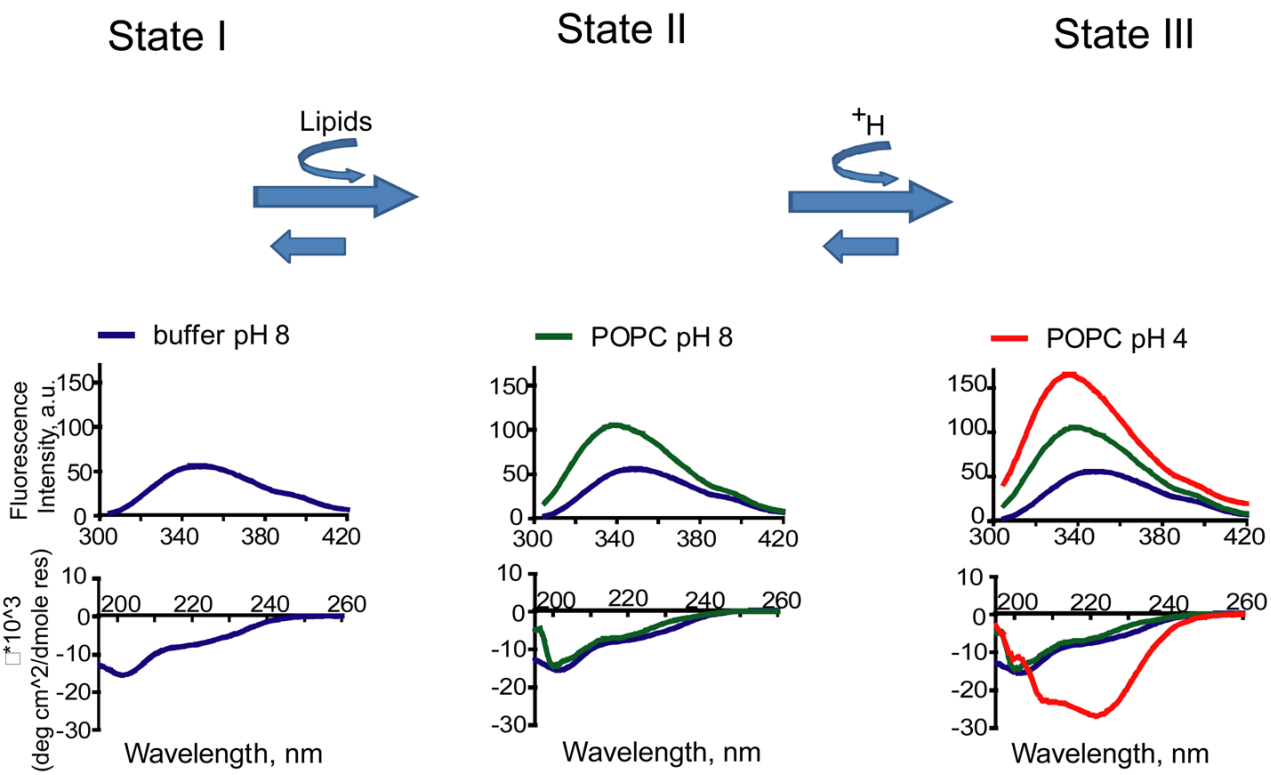

Figure 1.

A schematic representation of the three principal states of WT-pHLIP. State I: pHLIP is unstructured and soluble in water at neutral pH, State II: pHLIP is unstructured and associated with the bilayer surface, State III: pHLIP is inserted across a lipid bilayer as an alpha-helix. Tryptophan fluorescence and CD spectra of the WT-pHLIP in each state are shown. A plot corresponding to each state is added to the graph of the previous state. The fluorescence and CD spectra corresponding to each state, together with the sketch of the peptide in each state is color coded as follows: State I-blue, State II-green, State III-red. See text for detailed explanations. Membrane models were obtained from RasMol images of lipid bilayers (26).

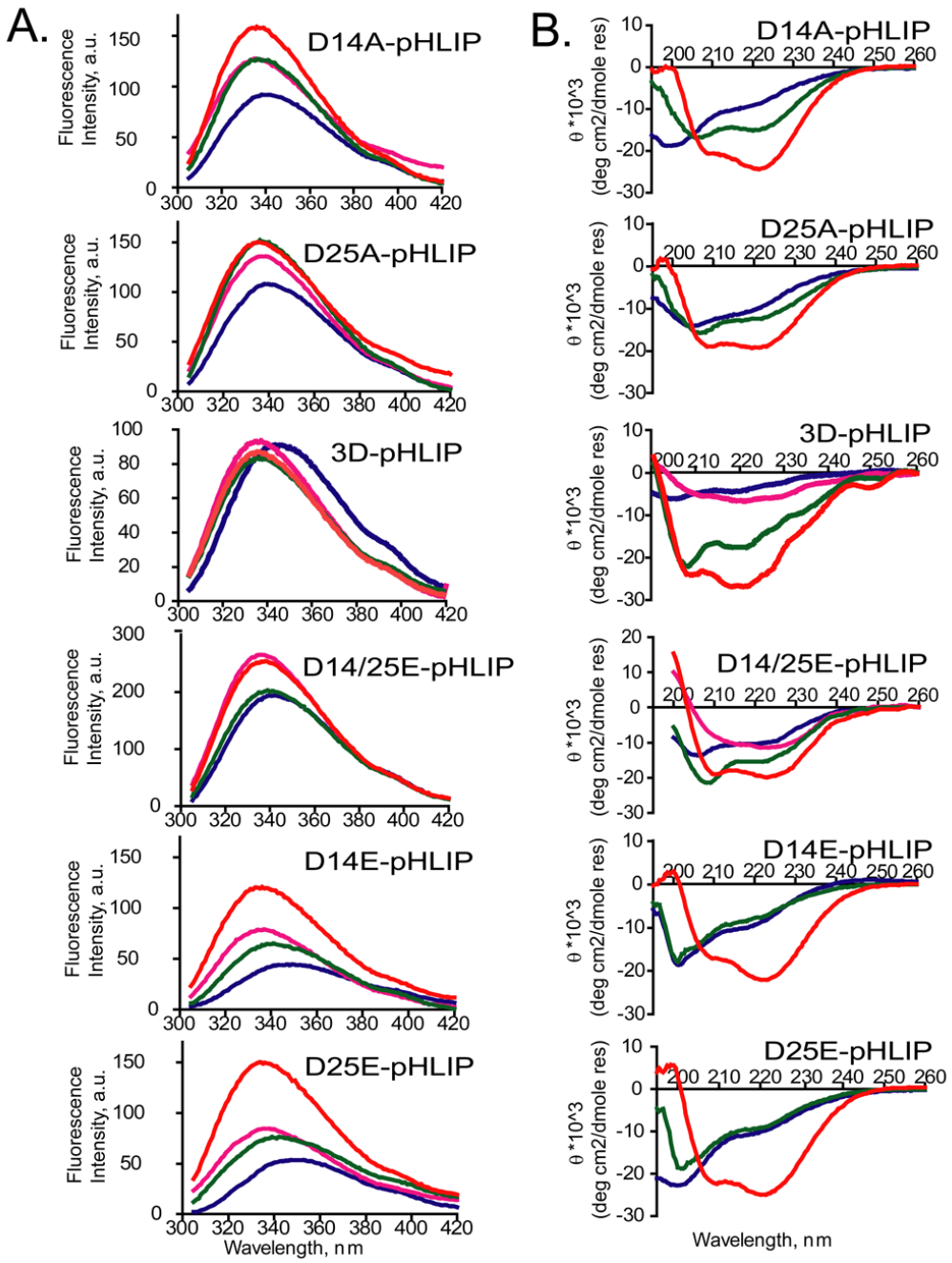

Figure 2.

Tryptophan fluorescence (A) and CD (B) spectra of pHLIP variants in solution at pH 4 and 8 in the absence and presence of POPC vesicles are presented. The concentration of pHLIP variants and POPC vesicles are 7 μM and mM, respectively. Blue and pink lines represent peptides in phosphate buffer at pH 8.0 and pH 4.0, respectively; green and red lines represent peptides in the presence of POPC vesices at pH 8.0 and pH 4.0, respectively. The positions of fluorescence spectra maxima, L_{max}, are given in Table 3.

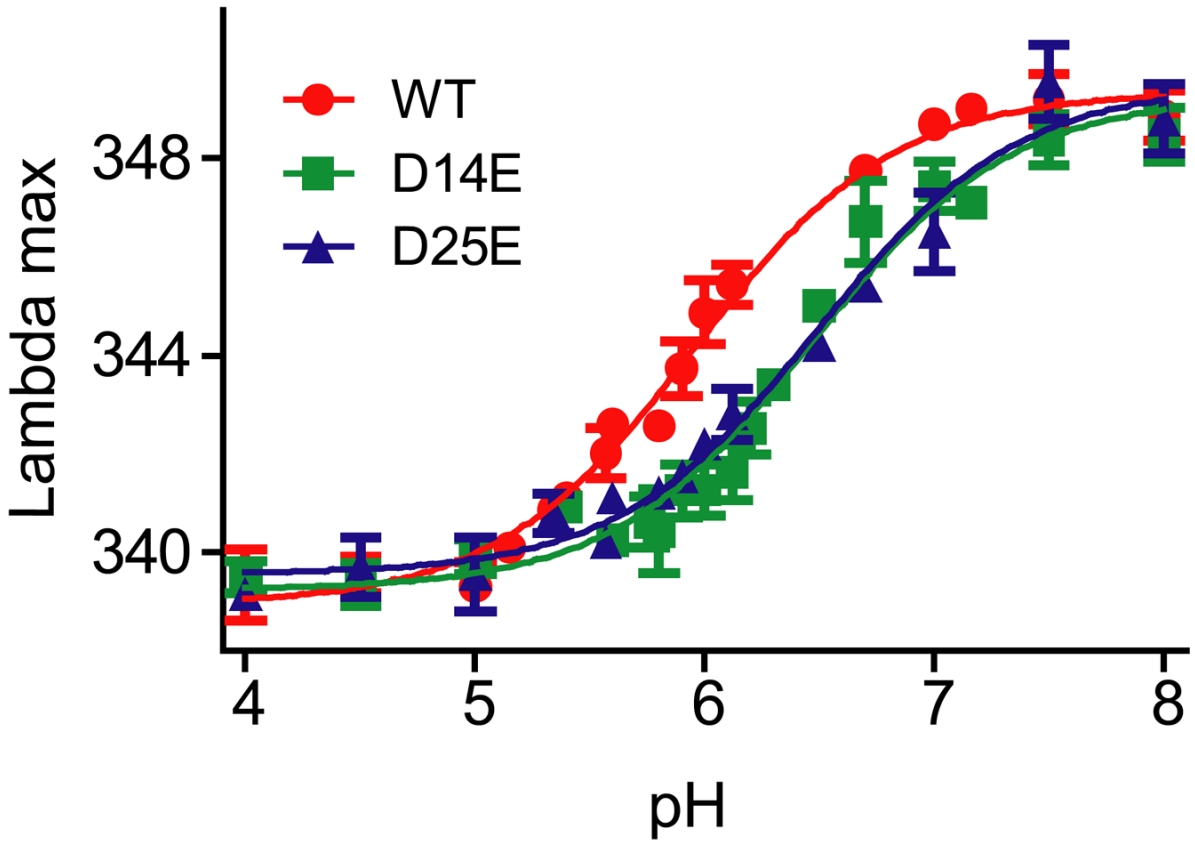

Figure 3.

pH induced insertions of pHLIP and two D to E variants across a lipid bilayer are shown. The titration was monitored by the shift of tryptophan fluorescence spectra maxima of pHLIP peptides in the presence of POPC in response to a decrease of pH from 8.0 to 4.0. The values of the position of fluorescence spectra maxima are the averages of at least three independent measurements ± standard errors. The curves represent the best-fits of the experimental data using the GraphPad Prism software. pK_a insertion values of pHLIP variants calculated from the plots are shown in Table 4.

Table 1

Sequences of pHILIP variants.*

pHLIP	Sequnece
WT	AAEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT
D14A	AAEQNPIYWARYAAWLFTTPLLLLDLALLVDADEGT
D25A	AAEQNPIYWARYADWLFTTPLLLLALALLVDADEGT
D14E	AAEQNPIYWARYAEWLFTTPLLLLDLALLVDADEGT
D25E	AAEQNPIYWARYADWLFTTPLLLLELALLVDADEGT
D14/25E	AAEQNPIYWARYAEWLFTTPLLLLELALLVDADEGT
3D	AAEQNPIYWARYADWLFTDLPLLLLDLLALLVDADEGT

*TM segment (based on the bacteriorhodopsin structure) is highlighted in blue and changed or inserted residues are marked in red.

Table 2

The apparent free energy difference (ΔG_{app}) calculated for the potential TM helices of pHLIP variants.*

	$\Delta G^{aa(i)}_{app}$†	$\Delta G_{hyd.mom}$	ΔG_{length}	ΔG_{app}
WT	+0.13	+0.39	0.00	+0.53
D14A	−0.59	+0.21	0.00	−0.38
D25A	−1.14	+0.21	0.00	−0.92
D14E	+0.28	+0.43	0.00	+0.71
D25E	0.00	+0.37	0.00	+0.37
D14/25E	+0.15	+0.40	0.00	+0.55
3D	+0.33	+0.64	−0.32	+0.66

*Prediction of ΔG for TM helix insertion was based on the ΔG prediction server v1.0 found at http://www.cbr.su.se/DGpred/index.php?p=Tmpred. A negative value of ΔG_{app} indicates that the sequence is predicted to be recognized as a TM helix by the Sec translocon and integrated into the membrane, a positive value of ΔG_{app} does not necessarily mean that the peptide is not a transmembrane helix.

†$\Delta G^{aa(i)}_{app}$ is the contribution from amino acid *aa* in position *i*.

Table 3

Position of tryptophan fluorescence spectra maxima (L_{max}) of pHLIP variants in the absence and presence of POPC at pH 8 or pH 4.*

pHLIP	Buffer		POPC	
	pH 8	pH 4	pH 8	pH 4
WT	349 nm	336 nm	343 nm	336 nm
D14A	342 nm	335 nm	337 nm	336 nm
D25A	339 nm	337 nm	336 nm	336 nm
3D	344 nm	336 nm	337 nm	336 nm
D14/25E	342 nm	336 nm	339 nm	337 nm
D14E	349 nm	337 nm	341 nm	336 nm
D25E	349 nm	336 nm	342 nm	336 nm

* L_{max} values correspond to the numbers obtained from fluorescence curves in Figure 1 for the WT-pHLIP and Figure 3A for pHLIP variants.

Table 4

Insertion pK_a values of WT-pHLIP and its D to E variants across a membrane, calculated from the fitting of the titration curves obtained by measurement of tryptophan fluorescence spectra of peptides in the presence of POPC vesicles at different pHs.

pHLIP Variants	WT	D14E	D25E
pK_a	5.96 ± 0.05	6.46 ± 0.08	6.49 ± 0.08