Published in final edited form as:

Biochemistry. 2013 July 9; 52(27): 4595–4604. doi:10.1021/bi400252k.

Aspartate Embedding Depth Affects pHLIP's Insertion pKa

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Abstract

We have used the pHLIP® (**pH** **L**ow **I**nsertion **P**eptide) peptide family to study the role of aspartate embedding depth in pH-dependent transmembrane peptide insertion. pHLIP binds to the surface of a lipid bilayer as a largely unstructured monomer at neutral pH. When pH is lowered, pHLIP inserts spontaneously across the membrane as a spanning α -helix. pHLIP insertion is reversible when pH is adjusted back to a neutral value. One of the critical events facilitating pHLIP insertion is the protonation of aspartates in the spanning domain of the peptide: the negative side chains of these residues convert to uncharged, polar forms, facilitating insertion by altering the hydrophobicity of the spanning domain. To further examine this protonation mechanism, we created pHLIP sequence variants in which the position of the two spanning aspartates (D14, D25) was moved up or down in the sequence. We hypothesized that aspartate depth in the inserted state would directly affect the proton affinity of the acidic side chains, altering the pKa of pH-dependent insertion. To this end, we also mutated the arginine at position 11 to see if arginine snorkeling modulates the insertion pKa by affecting aspartate depth. Our results indicate both types of mutations change the insertion pKa, supporting the idea that aspartate depth is a participating parameter in determining pH dependence. We also show that pHLIP's resistance to aggregation can be altered with our mutations, identifying a new criterion for improving pHLIP performance *in vivo* when targeting acidic disease tissues such as cancer and inflammation.

Keywords

alpha helix; peptide folding; membrane insertion; tumor acidity; bacteriorhodopsin

We have shown previously that the C helix of bacteriorhodopsin, called pHLIP® (**pH** **L**ow **I**nsertion **P**eptide), is capable of targeting acidic tissues and inserting into cell plasma membranes both *in vitro* and *in vivo*^{1, 2, 3, 4}. This is because localized extracellular acidity is a hallmark of many diseases including cancer, inflammation, and ischemia.²⁶ When conjugated to PET⁵ and fluorescent^{1, 4} probes, pHLIP is able to target mouse tumors *in vivo* with high specificity, demonstrating potential for therapeutic use in cancer diagnosis⁶.

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Supporting information available:

Data: 1) insertion reversibility experiments, 2) ionic and pH aggregation challenges, 3) comparative summary tables of circular dichroism and tryptophan fluorescence data, 4) fluorescence spectral maxima vs. pH plots for double variants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

pHLIP may also function as a drug delivery platform, translocating small cell-impermeable cargo molecules such as organic dyes², cyclic peptides^{3, 21}, and peptide nucleic acids (PNAs) into the cytoplasm of cultured cells². Importantly, pHLIP does not exhibit obvious toxicity in cells or mice^{1, 4}, further promoting its therapeutic potential.

pHLIP is monomeric at low concentrations⁷, adopting a largely unstructured conformation in neutral pH aqueous environments^{8, 23}, a condition we call state I. When lipid vesicles are added at neutral pH, pHLIP partitions favorably to the lipid surface with a ΔG of 6–7 kcal/mol⁹. In this surface-attached state, called state II, pHLIP remains largely unstructured^{8, 11}, hardly distinguishable from state I by circular dichroism. However, when the pH is lowered, pHLIP inserts into membranes as a membrane-spanning α -helix^{7, 22}, adopting the state III conformation. This insertion is fully reversible with the peptide's C-terminus translocated across the membrane upon insertion^{3, 22}. The pKa of insertion into POPC liposomes is 6.0^{8, 11}.

The pHLIP primary sequence contains several acidic residues (Table 1). At neutral pH, the combined negative charge of these residues, together with the free carboxyl at the C-terminus, constitute a large energy barrier, preventing pHLIP insertion at neutral pH. The energetic cost of partitioning a single aspartate from water into octanol by the Wimbley-White scale is 3.6 kcal/mol for the unprotonated form but only 0.4 kcal/mol for the protonated form¹⁰. It is this conversion that allows pHLIP insertion at low pH^{1, 7}.

Previous work has shown the number of aspartic residues in the inserting portion of the peptide has an effect on pHLIP's insertion pKa¹¹. In the following work, we examine whether the *position* of the two spanning aspartates at positions 14 and 25 play a role as well. Local water availability around acidic residues is known to play an important role in determining the proton affinity of the side chains¹². We were therefore interested in determining if changes in position would give rise to changes in insertion pKa, indicating embedding depth in the inserted state (state III) affects pHLIP's pH-sensitivity by modulating proton affinity. On related lines, we were interested in seeing if mutating the arginine at position 11 exhibited any effects on the insertion pKa, since changes could indicate arginine snorkeling also participates in regulating aspartate embedding depth in state III.

Materials and Methods

Peptide Synthesis

Peptides were made using the 9-fluorenylmethyloxycarbonyl solid-phase synthesis service provided by the W.M. Keck Foundation Resource at Yale University (New Haven, CT). After synthesis, peptides were purified by reverse phase chromatography on a C18 column using a water/acetonitrile gradient. Purity was determined by MALDI-TOF mass spectrometry and peptide quantity was determined by absorbance spectroscopy, using a molar extinction coefficient of 13940 M⁻¹ cm⁻¹.

Peptides were synthesized with a single cysteine residue at the C-terminus for forming disulfide bond cargo attachment (see Table 1 for list of variants). To work out ionic strength

conditions that did not promote dimerization or aggregation, different amounts of WT pHLIP-cys were incubated in phosphate buffer for varying periods of time. The resulting samples were assayed for dimer by both HPLC (data not shown) and tryptophan fluorescence (Fig. 1A, data not shown). Both WT pHLIP disulfide dimerization (reagent-assisted) and aggregation (at high peptide concentrations) have been shown to cause a quenching blue shift detectable by fluorescence. These experiments yielded HPLC and tryptophan fluorescence data in agreement with each other, showing that very low concentrations of WT pHLIP ($\sim 7 \mu\text{M}$) do not dimerize or aggregate significantly for very long periods of time (>12 hours) in 5, 10, and 20 mM phosphate buffer. Because of the agreement between assays and since HPLC requires more material, only tryptophan fluorescence was used to assess aggregation/dimerization in the pHLIP variants.

Peptide preparation

The final peptide preparation procedure used for this work involved resuspending lyophilized peptide flakes in 5 mM phosphate buffer at pH 8.0 to create peptide stocks in the range of 20–30 μM . These stocks were used within one hour to avoid aggregation.

Liposome preparation

The desired amount of chloroform-dissolved POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid from Avanti Polar Lipids was dried in a glass tube, first with nitrogen gas and then under vacuum overnight. The dried lipid was resuspended in 5 mM phosphate buffer, pH 8.0 to achieve the desired stock concentration and vortexed to mix thoroughly. To make POPC liposomes, the resuspended lipids were extruded through one polycarbonate membrane of 0.1 μm pore size (Nuclepore from Whatman). This extrusion was performed using a “Mini-Extruder” syringe system. Depending on the lipid concentration, 15–30 extrusion steps were performed to obtain a homogenous population of large unilamellar vesicles (LUVs). HPLC was used once when establishing the procedure to determine a homogenous vesicle population.

Insertion Assay by Tryptophan Fluorescence

Peptide and POPC vesicle stocks were prepared as described above, diluted, and incubated together at a molar peptide:lipid ratio of 1:300 for 1 hour. The dilution was in 5 mM phosphate buffer, pH 8.0, reaching a final peptide concentration of 10 μM . This incubation was performed to give the peptide ample time to complete partitioning to the liposome surface (the “parking step”).

After incubation, the peptide/vesicle stock was further diluted to create a panel of samples, each at a final peptide concentration of 1.5 μM . The pH was adjusted in this final dilution using a panel of 250 mM phosphate stock buffers, each titrated to a different pH. Each sample in the panel was mixed with a fixed aliquot of stock buffer so that the pH was adjusted to a unique value in the range from 3.5 to 8.0 while maintaining constant buffer concentration. 20–30 samples were made to provide adequate coverage of the pH range. The final phosphate concentration was 20 mM. After a 10–15 minute incubation to allow the pH titration to take effect, the emission spectrum of each sample was measured at room

temperature with excitation at 295 nm using a SLM-Aminco 8000C spectrofluorimeter. Appropriate liposome-only blanks were subtracted in all cases.

For the determination of the spectral maxima, a fitting procedure using the root-mean-square criterion (SIMS) was applied to the emission curves. This procedure fits the experimental spectra to a series of log-normal components²⁵. The spectral maxima thus obtained for each sample in the pH range was plotted and analyzed according to a sigmoidal fit¹⁸ as follows:

$$F = \frac{(F_a + F_b 10^{m(pH-pK_a)})}{(1 + 10^{m(pH-pK_a)})} \quad (\text{Equation 1})$$

where $F_a = (f_A + S_A \text{ pH})$ and $F_b = (f_B + S_B \text{ pH})$; f_A and f_B are the spectral maxima for the acid and basic forms, respectively, and S_A and S_B are the slopes of the acid and base baselines. The midpoint of the sigmoid gives the pKa of insertion. Fitting by nonlinear least squares was carried out using the Origin software (Origin Lab, MA).

Insertion Assay by Circular Dichroism

Samples were prepared as in the tryptophan fluorescence experiments but with a final molar peptide:lipid ratio of 1:200 and a final peptide concentration of 5 μM . This was done to both enhance the signal from the peptide and reduce light scattering from the lipids. CD spectra were recorded on a Jasco J-810 spectropolarimeter interfaced with a Peltier temperature system. Spectra were recorded at room temperature using a 2 mm cuvette. The scan rate was 100 nm/min and 30 scans were averaged for each sample. Raw data was converted to mean residue ellipticity as follows¹³:

$$[\theta] = \frac{\theta_{obs}}{10 \cdot l \cdot c \cdot n} \quad (\text{in degrees cm}^2 \cdot \text{dmol}^{-1}) \quad (\text{Equation 2})$$

where Θ is the measured ellipticity, l is the cell path-length, c is the protein concentration, and n is the number of amino acids per molecule. Appropriate lipid blanks were subtracted in all cases.

State I Aggregation Experiments

Peptide stocks for ionic and pH aggregation were prepared as above. In ionic aggregation experiments, the peptide stock was diluted into a series of samples adjusted to a different final ionic strength by varying volumes of 250 mM phosphate buffer, pH 7.4. The final peptide concentration for all samples was 3 μM and the final pH of the samples was in the range of 7.44 ± 0.10 . Linear fits were made using Excel.

For pH aggregation experiments, the peptide stock was diluted into a series of samples adjusted to a different pH by a panel of stock 250 mM phosphate buffers. The resulting series had a peptide concentration of 3 μM and phosphate buffer concentration of 10 mM for a final pH between 4 and 8. Midpoints were determined using the same fitting as above (equation 1).

After preparation, each set of samples was incubated for 10 minutes before fluorescent measurement at room temperature as described above. Time was an important variable for the output of these experiments so the incubation time had to be controlled. For ionic aggregation, samples were always measured from lower ionic strength to high while pH variation samples were measured from high pH to low.

Reversibility Experiments

Peptide and lipid stocks were prepared as described above. Peptide/lipid mixtures were made from these stocks in 5 mM phosphate buffer, pH 8.0 as for fluorescence. The peptide:lipid ratio was 1:300 and the peptide concentration was 5 μ M. After an hour incubation to “park” the peptides, samples were read once by fluorescence before the pH was adjusted to a value of 4.11 ± 0.09 using a set quantity of concentrated HCl. The samples were read again by fluorescence over a course of 10 minutes before a small aliquot ($< 3 \mu$ l) of concentrated sodium hydroxide was added to return the pH to 7.69 ± 0.07 . The tryptophan fluorescence was again read across a 10 minute interval. Spectral maxima were determined as described above.

Results

Selection of Single Mutant Variants

It has been shown previously that the proton affinity of acidic side chains depends on the local water environment¹² such that a more hydrophobic environment results in higher affinity. This mechanism depends on the fact that there are fewer water molecules to solvate the acidic proton once it is bound to the side chain.

In the context of inserted pHLIP (state III), it is conceivable that the embedding depth of the spanning aspartates at positions 14 and 25 in state III could play a role in determining the off-rate of the acidic protons. Previous work¹⁴ has shown the water profile inside a lipid bilayer is uneven with hydration decreasing precipitously with deeper penetration. This lack of water could mean side chain-proton affinity is higher at locations deeper in the membrane.

The most direct way of examining embedding depth as a parameter is to directly change the position of the aspartic residues in the spanning domain. This is the first of two strategies we employed. The second, more indirect strategy involved mutating the arginine at position 11 (R11). It has been reported previously^{15, 16} that arginines can have strong “snorkeling” effects that buoy regions of a transmembrane peptide closer to the interfacial surface. An open question for pHLIP was whether or not R11 plays such a role. To examine the possibility, we mutated R11 to a glutamine (Table 1). A glutamine was selected because its hydrophobicity was most similar to arginine by the Wimley-White scale¹⁷.

Defining Experimental Conditions with WT pHLIP

To ensure experimental consistency, we defined a specific set of conditions with WT pHLIP. Previous work^{2, 8} has established pH, peptide concentration, and peptide to lipid ratio guidelines for performing pHLIP experiments. However, one parameter not well

established until now is ionic strength. Previous protocols have involved dialyzing pHLIP into solutions of physiological ionic strength¹⁶. However, it has been found that exposure to high ionic strength results in both fluorescence blueshift and loss of tryptophan fluorescence intensity (Fig. 1A, C). This is consistent with previous observations of WT pHLIP in which aggregation or dimerization of the peptide at high concentration, confirmed by HPLC, produced an identical fluorescent effect (unpublished data). Because of these observations, we devised a peptide preparation protocol with a very low peptide concentration and low ionic strength to prevent aggregation/dimerization. Both restrictions alleviated the problem significantly when using WT peptide (Fig. 1 and data not shown).

Interestingly, it was found that ionic strength had an effect on the spectral maxima of pHLIP samples in state II (Fig. 1A) as well as state I. This suggested that increasing ionic strength promotes partitioning of pHLIP from the aqueous environment to the membrane surface. This would be consistent with the mechanism of soluble charge driving hydrophobic components away from solution: something that should promote membrane partitioning and aggregation similarly. It was not surprising, therefore, to find different ionic conditions resulting in slightly different insertion pKa values as well (Fig. 1D) since state II partitioning is an integral part of insertion. These results are significant when considering future use of WT pHLIP and pHLIP variants *in vivo* since the ionic strength of blood is considerably higher than these optimized values *in vitro*.

Tryptophan Fluorescence

In addition to indirectly assessing aggregation, tryptophan fluorescence (TF) can be used to monitor the local environment of tryptophan residues at positions 9 and 15 (Fig. 2A), giving direct information about pHLIP's interactions with membranes through assessment of the local hydrophobic environment. To test our variants for membrane interaction, we performed insertion experiments into POPC liposomes using low-ionic conditions. Much to our surprise, not every peptide exhibited three distinguishable states like WT. The D25Up and D25Down variants, for instance, exhibited state II behavior that looked more similar to WT state III (Fig. 2E–F). This indicated the possibility the peptides were either inserting spontaneously or aggregating on the membrane surface. D14Down also exhibited this aberrant state II behavior but to a lesser extent (Fig. 2D). As the sequence variations from WT pHLIP are very small and the overall hydrophobicity is identical, it seems most likely the membrane surface is inducing aggregation since there would seem to be no other element in the system to overcome the large energy barrier for insertion.

From plots of the tryptophan spectral maxima across pH in the presence of liposomes, it is possible to reconstruct the state II to III transition as a sigmoid for WT pHLIP (Fig. 3A). Using this method, we can mathematically determine the midpoint, which is the pKa of insertion. This pKa defines the acidic proton concentration at which half of all peptide is inserted into the membrane⁸. In agreement with our hypothesis of D25Up and D25Down as aggregating, no insertion pKa was obtained for these peptides (Fig. 3B). The remaining peptides, with the exception of D14Down, all had insertion pKa values lower than that of WT pHLIP.

Circular Dichroism

Circular dichroism (CD) is used to examine the secondary structure of peptides. We employed it to monitor the helical transition of state II to III (Fig. 4A). Like the fluorescence, D25Up and D25Down exhibited altered state II behavior, showing more secondary structure than WT (Fig. 4E–F). In addition, their state I seemed to have more helicity, another strong indicator of aggregation. D14Down similarly exhibited state I and state II behavior very different from WT but not as different as D25Up and D25Down. This suggests D14Down aggregates only partially (Fig. 4D). All other variants showed behavior similar to WT. Some effort was put into trying to reconstruct insertion pK_a values from CD plots but the variability of data from experiment to experiment proved to be a deterrent (data not shown).

Insertion Reversibility

An important criterion for true pHLIP insertion is reversibility. WT pHLIP insertion can be reversed by raising the acidic pH of a state III sample above 7 (Fig. S1A). To examine reversibility in our variants, the fluorescence from peptide/POPC samples was recorded once at pH 7.8, recorded again over a 10-minute time interval after adjusting the pH to a low value (pH 4.11±0.11), and recorded again over a second 10-minute interval after the pH was returned to a basic value (pH 7.69±0.10). As might be anticipated from the other experiments, R11Q and D14Up exhibited clear reversibility much like WT (Fig. S1A) while D14Down exhibited some reversibility, consistent with the idea it may be only partially aggregating. D25Up and D25Down exhibited no reversibility, again consistent with aggregation (Fig. S1B).

Aggregation by Ionic Strength Challenge

Since pHLIP's aggregation is intimately related to ionic strength (Fig. 1A), we were curious to see how the variants would respond to an ionic strength challenge. For use *in vivo*, pHLIP-fluorophore conjugates are typically injected into mice at very high concentrations (30–100 μM). Since body fluids are of moderately high ionic strength (similar to phosphate buffers at 50–55 mM), it remains questionable how much of the injected peptide is actually in a functional, monomeric form. It is possible serum albumin and other components of the blood help to dissociate aggregates, but it is also possible that pHLIP aggregates are permanent, significantly reducing the available pool of functional monomer. From the standpoint of clinical use, it is therefore desirable to engineer peptides that have as little aggregation as possible while maintaining pH-dependent insertion.

By plotting the spectral maxima of peptide samples across a phosphate buffer concentration range, we were able to observe three types of behavior (Fig. S2A). For D25Up, and D25Down, the spectral maxima blue shifted rapidly before saturating. WT and D14Down also exhibited saturation behavior but more gradually and at higher ionic strengths. The spectral maxima of the remaining peptides behaved linearly. From these plots, we fit an initial slope to each (Fig. S2B), using this value as an indication of how quickly each peptide responds to ionic challenge. Interestingly, R11Q aggregated less than WT pHLIP, suggesting it may be more amenable for use *in vivo* while still maintaining pH-dependent

insertion. In addition to aggregating less with an ionic challenge, R11Q also did not saturate in the ionic range examined (Fig. S2A).

Aggregation by pH Challenge

Although not as physiologically relevant as ionic aggregation, we were also interested in seeing how the variants responded to pH challenge. We knew from previous, unpublished work that pHLIP aggregates readily in solution below pH 6.0 in the absence of lipids. By plotting the spectral maxima across a pH range, we found pH challenge resulted in sigmoidal transitions much like those observed for lipid insertion. Taking the midpoints of these sigmoids as the “pH aggregation pKa” we found that the pattern of pH dependence was the same as for ionic challenge: D14Down, D25Up, and D25Down aggregated at a higher pH than WT while R11Q and D14Up responded at a lower pH (Fig. S3A). Interestingly, when plotting these pH-dependent aggregation midpoints alongside the insertion pKa values, we found that each aggregation pKa was lower than the corresponding insertion pKa (Fig. S3B). We speculate this may be a characteristic requirement for peptides that undergo a true pHLIP-like state II to III transition: they must have higher pH-dependent affinities for lipids than themselves.

R11Q;D14Up Double Variant

From the above data, it might be hypothesized that a pHLIP double variant with both R11Q and D14Up might have a combination of desired properties: a lower insertion pKa than WT and less aggregation. To test this notion, we created the R11Q;D14Up double variant (Fig. 5A) and found it had a lower insertion pKa than the two single variants (Fig. 5B, S6) and less aggregation when challenged with ionic strength (Fig. S4A). pH-dependent aggregation (Fig. S4B) was not obviously different from R11Q, but was lower than D14Up. This variant still exhibited clear, reversible (Fig. S4C) insertion by fluorescence and CD (Fig. 5C and 5E). We suggest this variant may prove useful for *in vivo* applications for reasons discussed above.

R11Q;D14UpUp Double Variant

To explore further the idea of aspartic depth in state III as a parameter for controlling the insertion pKa, we also created the R11Q;D14UpUp variant. This variant was one in which the R11Q mutation was combined with a new mutation in which the aspartate at position 14 was moved up two units in the primary sequence (Fig. 5A). If aspartate depth was directly responsible for the lower pKa of the R11Q;D14Up variant, we expected the R11Q;D14UpUp variant to have an even lower pKa.

Interestingly, a lower pKa was not observed. Although fluorescence and CD indicated a peptide that exhibited proper pH-dependent insertion (Fig. 5D and 5F), R11Q;D14UpUp had a pKa indistinguishable from that of D14Up (Fig. 5B, S6), despite exhibiting insertion reversibility (Fig. S4C) and aggregation behavior very similar to that of R11Q;D14Up (Fig. S4A–B).

Discussion

The two main outcomes from this study are: 1) further understanding of the elements of pHLIP's primary sequence that control its aggregation properties and insertion pKa and 2) the possible design of a peptide with improved properties for use *in vivo*. For the latter, a reduced aggregation tendency and lower insertion pKa are likely desirable properties. Reduced aggregation tendency may result in a larger pool of conjugated peptide that is available for targeting while a lower insertion pKa may enhance targeting by increasing sensitivity to local acidity, reducing off-target localization. This reduction is especially important since the pH difference between normal tissue and tumor tissue is often quite small: typically a difference of 0.2 to 0.5 pH units depending on experimental method and tissue type.²⁶

As the results above show, the D14Up, R11Q, R11Q;D14Up, and R11Q;D14UpUp peptides exhibit both a lower insertion pKa than WT and superior resistance to aggregation. From a pure materials standpoint, we offer R11Q;D14Up as the peptide with greatest potential for improved *in vivo* use. And although the potential for any enhanced localization *in vivo* is not explored in this study, future work should examine the possibility.

In the aberrant cases of D14Down, D25Down, and D25Up, a direct assessment of what is happening (relative to WT) is problematic. Given the strong blue shift of all three peptides in state II, we speculate they are most likely aggregating at the membrane surface. It is very interesting to note how drastically the properties of the peptide can change, even with a single one-position modification in sequence order. This offers the tantalizing possibility that D25 may be part of a critical interaction interface for the peptide in state II, an interface that can promote aggregation when D25 is moved out of the way. This is particularly interesting since previous work mutating D25 to histidine showed only very mild changes in behavior.¹¹

Insertion pKa Changes

One question we posed was whether the depth of the spanning aspartates played a role in changing the insertion pKa, as might be expected from the changing water profile in a bilayer²⁴. When examining the data, there is some support for this idea: when D14 is moved up, the insertion pKa decreases and when D14 is moved down (ignoring the partial aggregation), the insertion pKa increases, just as our hypothesis predicts.

R11Q's contribution is unclear because we have no way of knowing for sure how the mutation changes the relative depth of the spanning aspartates. However, the pKa for R11Q;D14UpUp goes against the model. In R11Q;D14UpUp, we have moved aspartate 14 up two positions and yet the pKa has increased. There are two possible explanations for this (among others we will not discuss). The first is that the model is wrong. The second, more interesting possibility is that an aspartic residue at position 12 (D14UpUp) may be too high in the sequence to remain in the hydrophobic core during the inserted state. If this were the case, that aspartate may no longer have to retain its acidic proton to keep the peptide stably inserted. In effect, an aspartate at position 12 may no longer "participate" (or may participate to some lesser degree) in determining the insertion pKa.

This idea of insertion frame prompted us to consider the question of what might be the most favorable portion of pHLIP to reside in the hydrophobic core. Using previous results²⁰ to extrapolate the approximate thickness of a POPC hydrophobic core (from the second acyl chain carbon of one monolayer to the second acyl carbon of the other), we found that a pure α -helix segment of about 18 residues is sufficient to fully span the extrapolated core. Using the MPEx membrane domain predictor,¹⁰ we determined what part of pHLIP might be most likely to reside in a membrane-spanning domain of this length and found the following sequence to be the most favorable (underlined):

WT pHLIP: GGEQNPIYWARYADWLFTTPLLLDLALLVDADEGT

We were surprised by this prediction because it does not contain the arginine at position 11 even though its mutation affects the insertion pKa. This offers the possible suggestion that R11 may have more of a role in state II embedding and impart its effects on the insertion pKa indirectly without being a component of the spanning domain.

It is, of course, essential to note any algorithm such as MPEx is inherently imperfect since the tilt of the inserted helix, the exact number and identity of the inserted residues, and the rotational conformations of the inserted side chains will be composed of a grand ensemble of states no algorithm can hope to convey. Yet, it is still interesting to consider that the above prediction may have some semblance of the *average* of the ensemble. In this case, moving D14 up or down one unit in the sequence would not affect the composition of residues in the spanning domain on average, lending weight to the idea that a change in proton affinity due to depth, not hydrophobicity of the spanning segment, is responsible for the altered pKa we observe.

It is also interesting to see that moving D14 up *two* units would bring it *outside* the putative frame, exchanging it for a tyrosine (see below). This would result in an average hydrophobicity change of -1.14 kcal/mol (assuming a protonated aspartate), making the thermodynamics of the inserted segment more favorable, agreeing with the unexpected higher insertion pKa we observe for R11Q;D14UpUp.

R11Q;D14UpUp: GGEQNPIYWARDYAWLFTTPLLLDLALLVDADEGT

This concept of using simple hydrophobicity calculations of the putative spanning domain to correctly predict the change in direction of the insertion pKa is something we have also done in other recent work¹⁹ using membranes of different acyl chain length. In this other work, we found that MPEx results were also good predictors of pHLIP's insertion pKa in lipids of short to intermediate core thicknesses, leaving some hope that the semblance of an average may yet have practical use in our system.

Acknowledgments

FUNDING SOURCE STATEMENT:

This work was supported by the National Institutes of Health grants R01-GM073857-04 and R01-CA133890-02.

We would like to thank Dr. Ming An, Dr. Damien Thevenin, Dr. Monika Musial-Siwiek, Dr. Takemasa Kawashima, Dr. Miriam Alonso, Dr. Fred Gorelick, Dr. Michael Caplan, and Dr. Yorgo Modis for their time, support, and helpful discussions in the assembly of this work.

ABBREVIATIONS

pHLIP	pH Low Insertion Peptide
CD	circular dichroism
TF	tryptophan fluorescence
LUVs	large unilamellar vesicles
PET	positron emission tomography
HPLC	high-performance liquid chromatography
WT	wildtype

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Fig. 1. Ionic strength affects the three states of pHLIP

A) Increasing phosphate concentration blue shifts states I and II tryptophan fluorescence. 1.5 μ M WT pHLIP, 1:300 peptide:lipid ratio. **B)** Increasing [phosphate] increases state I helicity, presumably by driving aggregation: black = 5 mM phosphate, red = 25 mM, green = 50 mM, blue = 75 mM. 5 μ M pHLIP, pH 7.20 ± 0.09 . **C)** Increasing [phosphate] results in loss of state I fluorescence intensity, indicating tryptophan quenching or peptide loss from solution, both consistent with aggregation. Black = 5 mM phosphate, red = 20 mM, green = 40 mM, blue = 80 mM. **D)** Properties of a pH-dependent insertion sigmoid are changed with [phosphate]. Closed symbols are fluorescence spectral maxima at 20 mM phosphate, open symbols at 55 mM. The 20 mM insertion pKa = 5.95 ± 0.06 and 55 mM pKa = 6.18 ± 0.09 .

Fig. 2.

Fluorescence spectra of the three states of WT pHLIP (**A**), R11Q (**B**), D14Up (**C**), D14Down (**D**), D25Up (**E**), and D25Down (**F**). R11Q and D14Up exhibit a clear state II to III transition comparable to WT (see also Fig. S5). D14Down exhibits some II to III transition while D25Up and D25Down exhibit aberrant transitions. 1.5 μ M peptide and 0.45 mM POPC were used for all (a 1:300 peptide:lipid ratio). State I and II pH = 7.20 ± 0.10 , state III pH = 4.25 ± 0.08 .

Fig. 3. The variants have different insertion pKa

A) Fluorescence spectral maxima plotted across pH. 1.5 μ M peptide, 1:300 peptide:lipid ratio. **B)** The insertion pKa are different (average from $n = 4$). D25Up and D25Down were not plotted because they exhibit no obvious sigmoidal transition (from 3A).

Fig. 4. CD spectra of the three states of WT pHLIP (A), R11Q (B), D14Up (C), D14Down (D), D25Up (E), and D25Down (F). R11Q and D14Up exhibit obvious helicity increases between states II and III that mimic WT, in agreement with the fluorescence results (Fig. 2). D14Down exhibits a similar but different helical transition while D25Up and D25Down exhibit aberrant helicity in both states I and II (see also Fig. S5). 5 μ M peptide, 1:200 peptide:lipid ratio. State I and II pH = 7.22 $\pm$ 0.09. State III pH = 4.27 $\pm$ 0.08.

Fig. 5.

A) The sequences of the two double variants used (R11Q;D14Up and R11Q;D14UpUp), the parent sequences (R11Q and D14Up) and WT pHLIP. **B)** The insertion pKa of the double variants is compared to that of the parent variants and WT pHLIP ($n = 3$). **C–D)** Fluorescence of R11Q;D14Up (**C**) and R11Q;D14UpUp (**D**) under the three state conditions shows insertion behavior much like WT. 1.5 μM peptide was used at a 1:300 peptide:lipid ratio. **E–F)** CD of R11Q;D14Up (**E**) and R11Q;D14UpUp (**F**) under the three state conditions shows behavior much like WT: a clear helical transition between states II and III. 5 μM peptide was used at a 1:200 peptide:lipid ratio. (see also Fig. S5)

Table 1

Primary sequence of the pHLIP single mutation variants used in this study.^a

Name.	Sequence.
WT pHLIP	GGEQNPIYW RY AD W LF T TP L LL L D L ALLVDADEGTCG
R11Q	GGEQNPIYWA Q YADWLFTTP L LL L D L ALLVDADEGTCG
D14Up	GGEQNPIYWARY DA WLF T TP L LL L D L ALLVDADEGTCG
D14Down	GGEQNPIYWARYA WD LFTTP L LL L D L ALLVDADEGTCG
D25Up	GGEQNPIYWARYADWLFTTP LL DL ALLVDADEGTCG
D25Down	GGEQNPIYWARYADWLFTTP LL LD ALLVDADEGTCG

^aBlue letters indicate the arginine at position 11 and the aspartic acids at positions 14 and 25 that have been mutated. Red letters indicate the experimental alterations.