Roles of Carboxyl Groups in the Transmembrane Insertion of Peptides

Francisco N. Barrera¹, Dhammika Weerakkody², Michael Anderson², Oleg A. Andreev², Yana K. Reshetnyak² and Donald M. Engelman¹*

¹Department of Molecular Biophysics and Biochemistry, Yale University, PO Box 208114, New Haven, CT 06520, USA
²Physics Department, University of Rhode Island, Kingston, RI 02881, USA

Received 6 July 2011; received in revised form 3 August 2011; accepted 5 August 2011
Available online 23 August 2011

Edited by J. Bowie

Keywords:
membrane protein folding; pHLIP; pH trigger; carboxyl titration; transmembrane helix

We have used pHLIP® [pH (low) insertion peptide] to study the roles of carboxyl groups in transmembrane (TM) peptide insertion. pHLIP binds to the surface of a lipid bilayer as a disordered peptide at neutral pH; when the pH is lowered, it inserts across the membrane to form a TM helix. Peptide insertion is reversed when the pH is raised above the characteristic pKₐ (6.0). A key event that facilitates membrane insertion is the protonation of aspartic acid (Asp) and/or glutamic acid (Glu) residues, since their negatively charged side chains hinder membrane insertion at neutral pH. In order to gain mechanistic understanding, we studied the membrane insertion and exit of a series of pHLIP variants where the four Asp residues were sequentially mutated to nonacidic residues, including histidine (His). Our results show that the presence of His residues does not prevent the pH-dependent peptide membrane insertion at ~pH 4 driven by the protonation of carboxyl groups at the inserting end of the peptide. A further pH drop leads to the protonation of His residues in the TM part of the peptide, which induces peptide exit from the bilayer. We also find that the number of ionizable residues that undergo a change in protonation during membrane insertion correlates with the pH-dependent insertion into the lipid bilayer and exit from the lipid bilayer, and that cooperativity increases with their number. We expect that our understanding will be used to improve the targeting of acidic diseased tissue by pHLIP.

© 2011 Elsevier Ltd. All rights reserved.

Introduction

Extracellular acidification is a hallmark of different pathologies, including cancer, inflammation, ischemic stroke, and atherosclerotic plaques. Acidosis might be a useful biomarker for diagnosis or treatment if means can be found to target tissue acidity. We have found that a peptide derived from helix C of bacteriorhodopsin,¹ named pHLIP® [pH (low) insertion peptide], is capable of targeting acidic tissues and inserting into the cell plasma membrane.² pHLIP is able to target mouse tumors in vivo with high specificity,² opening the possibility of its use for cancer imaging. Additionally, pHLIP has a promising therapeutic potential, as it is able to translocate cell-impermeable cargo molecules, such as organic dyes, peptides, peptide nucleic acids, and toxins, across the plasma membrane into the cytoplasm of tumor cells.²,³ pHLIP itself does not have obvious acute toxicity in cells³ or in mice.² pHLIP is monomeric at low concentrations, with a mostly unstructured conformation in neutral and...
In the membrane-attached state, respectively) so that the membrane and those that traverse the hydrophobic core of the membrane during insertion. Accordingly, we have studied both the pH-driven membrane insertion and the exit process for a series of peptides where the key aspartic acid residues are sequentially mutated.

### Results

Previous studies in our laboratories revealed that sequence variations in the TM region of pHLIP can disrupt the delicate balance that preserves its water solubility. For example, a simultaneous change in the two aspartic acid residues at positions 14 and 25 to the homologous glutamic acid (Asp14/25Glu) resulted in a loss of pH-dependent membrane insertion due to aggregation of the peptide in aqueous solution (we have recently developed new pHLIP variants with several Glu residues, which preserve pH-dependent properties; unpublished data). In order to reduce the likelihood that the introduced variations in the peptides used in this work could cause aggregation, we decided to follow a dual strategy to increase their water solubility: (i) we added an Asp tag to the N-terminus (noninserting end) to increase the number of charges in the molecule, which typically improves the solubility of hydrophobic peptides; this resulted in the replacement of the N-terminal sequence AAEQ with DDDED (Table 1); and (ii) we used the TANGO algorithm to define the region of the pHLIP sequence with the highest aggregation tendency and found this to be the stretch from residue 21 to residue 30 (coinciding with the most hydrophobic region of the peptide). We then mutated Leu26 to Gly, which greatly reduced the predicted aggregation tendency.

We incorporated these modifications into a series of pHLIP variants, where four aspartic acid residues were sequentially mutated to nonacidic polar residues. The aspartic acid residues at the C-terminus of the peptide that transitorily traverse the core of the membrane upon insertion (Asp31 and Asp33) were replaced with polar but uncharged asparagine residues. On the other hand, for the Asp residues that are located in the core of the membrane after insertion (in positions 14 and 25), histidine was chosen as the replacement residue, as it is expected to be partially charged at neutral pH (thus improving water solubility) while being only slightly polar in its uncharged state (the transfer energies from water to the bilayer interior are 0.43 and 0.11 kcal/mol for the neutral forms of Asp and His, respectively) so that the

<table>
<thead>
<tr>
<th>Table 1. Sequence of the peptides</th>
</tr>
</thead>
</table>
| wt | AAEQPIYWARAYDWFTTPLLHDLALLVDADEC
| D3a | DDDDNPPIYWARYADWFTPPPLHGLALLVDADECT |
| D3b | DDDDNPPIYWARYADWFTPPPLHGLALLVDADECT |
| D2 | DDDDNPPIYWARYADWFTPPPLHGLALLVDADECT |
| D1c | DDDDNPPIYWARYAWFTTPPLLHAGALLVNADECT |
| D0c | DDDDDNPPIYWARYAFTPPPLLHAGALLVNADECT |

* The pHLIP sequence is referred to as wt.
* The variant peptides are named by a D, followed by the number of aspartic acid residues in the TM and C-terminal regions. Two different D3 peptides (D3a and D3b) were studied, each with different TM aspartic acid residues mutated. The acidic residues that are expected to interact with the hydrophobic core of the membrane at some stage of the insertion process (Asp14, Asp25, Asp31, and Asp33; in red) were mutated to the polar residues marked in boldface. The TM region of pHLIP was predicted, using the octanol scale, to be located between Ile7 and Leu29 (marked with inverted blue triangles). The N-terminus and the C-terminus were not capped.

* A version of D2–D0 without cysteine was employed in the experiments, except in the biotin translocation assay.
insertion properties of pHLIP may not be altered. The peptides were named D0–D3 according to the number of aspartic acid residues present in the regions of interest (TM and C-terminus; the positively charged N-terminus is not expected to interact with the membrane). For the variants with three aspartic acids, two alternatives were studied: one that kept Asp14 (D3a peptide) and the other that kept Asp25 (D3b peptide).

We conducted experiments to test the state of the variants in solution, where pHLIP is largely found as an unstructured monomer. Sedimentation velocity experiments were conducted to determine the oligomerization state of the different peptide variants in aqueous buffer. Previous analysis of wild-type (wt) pHLIP (at 7 μM in 10 mM phosphate buffer and 100 mM NaCl, pH 8) showed that pHLIP is mostly monomeric, but a small oligomer population is observed (~6%). We performed our sedimentation velocity experiments under the same conditions, but without NaCl in the solution. For each peptide, we observed a peak with a sedimentation coefficient of 0.72±0.12 S (Table 2 and Fig. 1), which corresponds to a molecular mass of 3.4±0.8 kDa. This is in agreement with the expected monomer masses of the different peptides (4126 Da for wt and ~4300 Da for the different variants), with the differences being ascribed to shape effects from the extended peptide. In the case of D1 and D0, a minor peak with a sedimentation coefficient of 3.3±0.3 S was also observed. This component represents 5±2% of the total population, and its sedimentation coefficient corresponds to a molecular mass of 43 kDa (roughly consistent with the presence of an octameric or decameric particle).

Comparison of our results with the previous report for wt suggests that the presence of oligomers is reduced at lower ionic strength. For the particular

<table>
<thead>
<tr>
<th>Analytical ultracentrifugation</th>
<th>Fluorescence</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation coefficient</td>
<td>Spectral maximum (nm)</td>
<td>Area curve</td>
</tr>
<tr>
<td>State I</td>
<td>State I</td>
<td>State II</td>
</tr>
<tr>
<td>wt</td>
<td>0.80±0.17</td>
<td>347.7±0.6</td>
</tr>
<tr>
<td>D3a</td>
<td>0.67±0.08</td>
<td>349.9±0.1</td>
</tr>
<tr>
<td>D3b</td>
<td>0.66±0.09</td>
<td>349.1±1.0</td>
</tr>
<tr>
<td>D2</td>
<td>0.84±0.16</td>
<td>348.2±0.1</td>
</tr>
<tr>
<td>D1</td>
<td>0.88±0.18</td>
<td>346.2±3.6</td>
</tr>
<tr>
<td>D0</td>
<td>0.75±0.20</td>
<td>347.2±1.0</td>
</tr>
</tbody>
</table>

a The sedimentation coefficient for the peak corresponding to the monomer is shown.
b The spectral maxima were calculated with PFAST (see Materials and Methods).
c Averages and standard deviations are provided.

Fig. 1. Sedimentation velocities of the peptide variants. The apparent sedimentation coefficient distribution is shown as derived from the sedimentation velocity profiles of the peptides (7 μM) in 5 mM phosphate buffer (pH 8).
case of the D1 and D0 peptides, they seem to have a slightly higher oligomerization tendency in solution, but they are still 95% monomeric. Thus, our results suggest that all the peptide variants remain soluble and are essentially monomeric. For the rest of the experiments, we employed peptide concentrations (1.5–5 μM) lower than that used for sedimentation analysis (7 μM); thus, the level of oligomers present for D1 and D0 is expected to be lower.

Fluorescence spectra of the peptides in aqueous solution at neutral pH showed that, in all cases, the emission maximum is centered around 347–349 nm (Fig. 2, black lines, and Table 2), indicating that the two tryptophan residues of the peptides are largely exposed to aqueous solution, as in fully unfolded proteins, and consistent with the slightly low sedimentation coefficient. This finding represents an improvement over the previously studied Asp14/25Glu mutant peptide, where peptide aggregation shifts the emission maximum to 342 nm in buffer at pH 8. A similar fluorescence maximum was also observed for the Asp14/25Asn mutant under the same conditions. The presence of mostly unstructured species in aqueous solution for each of the studied peptides was confirmed by circular dichroism (CD) experiments, since the observed CD spectra were characterized by a minimum at 203 nm (Fig. 3, black lines), as observed for pHLIP in state I.

The two lipid-interacting states of the pHLIP variants were then examined: state II, where wt pHLIP is mostly unstructured and attached at the bilayer surface, and state III, where wt pHLIP forms a TM helix at low pH. Fluorescence experiments in the presence of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes revealed that for the two D3 variants, the characteristic fluorescence signatures for states II and III were evident: (i) in the presence of liposomes at neutral pH (Fig. 2, blue lines), the fluorescence emission maxima of the peptides were slightly shifted from 348.7±1.0 to 346.2±1.2 nm, accompanied by a small fluorescence increase (Table 2); and (ii) when the pH was lowered to pH 4, we observed a large fluorescence increase and a spectral blueshift to 336.2±1.1 nm (red lines), which are typically observed when the Trp side chain is buried in the membrane hydrophobic core. To complement the fluorescence data, we performed CD experiments under the same conditions (Fig. 3). The CD signature of the pHLIP membrane insertion process consists of the appearance of the characteristic signals associated with the formation of α-helix: minima at 208 and 222 nm and positive ellipticity at 190 nm. Both D3 variants showed spectral changes very similar to those observed for wt upon acidification. Thus, we concluded that replacement of one of the Asp residues in the TM region of the peptide does not lead to changes in the peptide's ability to interact with the membrane in a pH-dependent manner.

The D2 variant, where both Asp residues are replaced by His residues, also demonstrates a pH-dependent membrane interaction. However, the

![Fig. 2. Fluorescence spectra of peptides in buffer and with POPC vesicles. Emission spectra of each variant were recorded under the following conditions: buffer at pH 7.5 (black lines), POPC at neutral pH (blue lines), and POPC at pH 4 (red lines). The pH values for the different POPC samples at neutral pH were selected according to the midpoint and slope of the transitions shown in Figure 6: wt, pH 7.5; D3a, pH 7.5; D3b, pH 7.1; D2, pH 6.5; D1, pH 6.2; D0, pH 8. The peptide concentration was 1.5 μM, and the lipid concentration was 375 μM. Fluorescence intensity is given in arbitrary units (AU).](image-url)
Reduction of pH leads to the protonation of negatively charged groups at the 8th in the presence of POPC decreases in the pH range 8–6, with no significant changes in the spectral maximum at pH 8–7 and with a small shift to lower wavelengths at pH 6 (Fig. S1). The amount of the helical structure of D2 at neutral pH is slightly higher than those of wt and D3 (Fig. 2 and Table 2), while no change is seen in the pH range 8–6. As an explanation, we suggest that D2 partitions somewhat more deeply into the membrane lipid bilayer than wt and D3 at neutral pH values, since His residues are expected to be only partially charged at neutral pH values, enhancing the hydrophobicity of the peptide TM and its affinity for the lipid bilayer. The decrease in fluorescence signal in the pH range 8–6 might be attributed to the partial quenching of emission of at least one of the Trp residues by one of the partially protonated His residues. At the same time, at neutral pH values, the peptide C-terminus containing four negative charges (two Asp, one Glu, and the C-terminus) does not partition into the membrane, keeping the peptide at the membrane surface. A further drop of the pH to pH 3–4 is associated with a fluorescence spectral maximum blueshift, an increase in fluorescence intensity (Fig. 2), and the appearance of a more pronounced negative band at 222 nm on CD spectra (Fig. 3), which is usually an indication of peptide insertion into the bilayer. Reduction of pH leads to the protonation of negatively charged groups at the C-terminus and peptide insertion into the membrane. At the same time, we expect that protonation of His residues at low pH should occur; this might lead to the peptide’s exit from the lipid bilayer or, alternatively, the formation of a pore channel in the lipid bilayer, where positively charged His residues would be pointed toward the channel. Calcein encapsulation control experiments that rule out the formation of pores in the membrane by the D2 and D3 peptides were performed (Fig. S2). Thus, most probably, the pKₐ for the protonation of His is shifted to very low pH values when it is embedded in a lipid bilayer. We carried out fluorescence pH titrations to compare the behaviors of D2 and wt peptides at pH values lower than 3.5 (Fig. S3). While no fluorescence change was detected for wt at acidic pH values, we observed that an additional process was present for D2 (with an apparent pKₐ of 2.5), characterized by a fluorescence decrease and a redshift of the spectral maximum, which might be associated with peptide exit from the lipid bilayer.

To establish the orientation of each helix in the membrane, we performed oriented circular dichroism (OCD) measurements in which the light beam is oriented perpendicular to the planes of a stack of oriented lipid bilayers containing the peptides of interest. Theoretical calculations and experimental data indicate that helices oriented with axes parallel with the membrane surface (perpendicular to the incident light) give CD signals distinctly different from those of helices oriented across the bilayer.

Fig. 3. CD of peptides in buffer and with POPC vesicles. Far-UV CD spectra were recorded for all variants under different conditions: buffer at pH 7.5 (black lines), POPC at pH 7.4 (blue lines), and POPC at pH 4 (red lines). The reversibility of the insertion process was studied by raising the pH of the samples from pH 4 (broken blue line) to pH 7.4. Reversibility for D0 was not studied, as the ellipticity changes between the states at pH 7.5 and pH 4 were negligible. In all samples, the final peptide and lipid concentrations were 5 μM and 1.5 mM, respectively.
(parallel with the incident light). In the range of 190–240 nm, the peptide CD spectrum is dominated by $\pi-\pi^*$ and $n-\pi^*$ transitions. The $\pi-\pi^*$ transition in a helix splits into three components, one of which gives rise to a negative Gaussian band near 205 nm, with its electric transition dipole parallel with the helical axis. When the incident light propagates parallel with the helical axis, the electric field vector is orthogonal to the 205-nm $\pi-\pi^*$ dipole transition, and there is no interaction between the electromagnetic wave and the dipole, leading to the disappearance of the negative band at 205 nm in a CD spectrum. Thus, when the supported bilayers are oriented perpendicular to the light propagation, a helix with a TM orientation will have a CD spectrum that contains a positive 190-nm band and a negative 225-nm band. If the helix adopts a membrane surface orientation on the supported bilayer, then all transitions are seen, and the OCD spectrum is the same as for a peptide CD spectrum in solution, with randomly oriented helices. Our data clearly indicate that D2 adopts a TM orientation at pH 3.5—4.5, while increasing the pH leads to peptide exit and the appearance of a membrane surface orientation of the helix (Fig. 4). The OCD spectrum at pH 1.9 does not correspond to a TM helix. Thus, we conclude that the $pK_a$ of both or at least one of the His residues is significantly shifted from 6.3–6.9 to a lower value (2.5) due to their location at the bilayer interface in state II, emphasizing the important influence of bilayer surface properties on the $pK_a$ values of dissociating groups in interacting peptides. A similar trend was previously observed for peptides that insert into membranes via the deprotonation of His residues, although the magnitude of the $pK_a$ shift was smaller. However, large changes in $pK_a$ are typically observed when the side chains are in different environments, as the protonation of titratable amino acids depends on the dielectric properties of their environment. A fitting example of large $pK_a$ changes is found in the native environment of pHLIP, bacteriorhodopsin, where Asp14 and Asp25 have $pK_a$ values of 7.5 and 9, respectively, significantly higher than the $pK_a$ values of 3.7–4.0 found for fully solvated aspartic acid side chains.

D1 has one less Asp residue at the C-terminus than D2. The slightly larger blueshift of fluorescence emission (Fig. 2) and the higher content of helicity observed in the presence of POPC at neutral pH values (Fig. 3) could be associated with an even deeper position of the peptide in the membrane. Fluorescence spectral blueshift and intensity increase, together with an increase in ellipticity at 222 nm, occur upon acidification; this might indicate protonation of Asp33, Glu34, and the C-terminus, as well as peptide insertion into the lipid bilayer. The OCD spectrum obtained for D1 at pH 3.3 (Fig. 4) does not show a clear TM orientation of the helix: some decrease in ellipticity at 205–225 nm—which might indicate the existence of a mixture of TM and surface-parallel orientations of helices or the appearance of a significantly tilted TM helix—is observed. D0, in contrast to all other pHILIP variants described above, has a blueshifted maximum of fluorescence emission (Fig. 2) at neutral pH values in the presence of POPC, with a high content of helical structure (Fig. 3). Virtually no changes in spectral signal occur for D0 upon acidification (Figs. 2 and 3). The OCD data primarily reveal a surface orientation of the helix at low pH values (Fig. 4), as expected for a peptide with no aspartic acids.

To study the magnitude and directionality of the membrane insertion of the peptides, we used a biotin–avidin binding assay. A biotin moiety was attached to the C-terminus of each peptide. The level of binding to avidin was measured, and the protection of the biotin molecule from avidin interaction was used to assess the translocation of the peptide C-terminus into the liposome interior. The biotin moiety was linked to the C-terminal Cys of the peptides via a long polar polyethylene glycol (PEG) linker. The linker has a double purpose. It facilitates biotin access to the avidin binding site.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** OCD spectra of D2, D1, and D0 measured on oriented POPC-supported bilayers at neutral (blue lines) and acidic (red lines) pH values. The OCD spectrum of D2 at pH 1.9 was also recorded (purple line). The experimental spectra are corrected for the lipid background.
and—more critically for our experiments—helps to delineate between an intraliposomal location and an extraliposomal location of the biotin, since the polarity of the moiety makes a location inside the hydrophobic region of the bilayer unlikely. We quantified the amount of biotin that binds to avidin molecules present exclusively outside the liposomes (see Materials and Methods for details). We did not detect avidin binding to biotin for the D2 peptide at low pH (Fig. 5a) due to the biotin translocation across the membrane, which complements our data (suggesting complete insertion of this peptide across the lipid bilayer) and confirms that the directionality of insertion is the same as for wt. Only partial translocation and no translocation of biotin across the membrane were seen for D1 and D0, respectively (Fig. 5a), in agreement with our results indicating partial (or tilted) insertion and no insertion into the lipid bilayer of D1 and D0, respectively. Additionally, the translocation of biotin (which can be considered as a cargo) across the membrane does not appear to significantly hinder the membrane insertion of the peptides. This might be explained by its small size (526 Da) and its moderate polarity (log \( P = −1.4 \); see Materials and Methods for details), which are both well within the range of cargo properties that pHLIP has been reported to effectively translocate. However, as the biotin assay used here is responsive to changes in the level of binding to avidin present outside of the liposomes, we cannot rule out the possible influences of different processes such as peptide aggregation, although we have no reason to suspect them.

How does the number of carboxyl groups affect the \( pK_a \) and cooperativity of insertion? We monitored the pH-induced changes in the position of the fluorescence emission maximum of the peptides, which provide details about peptide insertion into the lipid bilayer, in the presence of POPC (Fig. 5). A plot of the positions of the spectral maxima follows a sigmoid behavior as a function of pH, corresponding to the transition between the interfacial state and the inserted state for all variants (except for D0). Fitting the experimental data provides the two main parameters that describe the insertion process: \( pK_a \) and cooperativity (\( m \)). The \( pK_a \) of membrane insertion obtained for wt pHLIP is \( 5.94 ± 0.09 \), which is in agreement with previous reports. For the different variants, shifts of the \( pK_a \) to lower values (\( \sim 1 \) pH unit for wt and \( \sim 2 \) pH units for D1) (Figs. 6 and 7b). Our data indicate that the cooperativity of insertion is linked to the number of protonatable residues. Cooperativity and \( pK_a \) might also respond to the position of protonatable groups in the peptide sequences and their proximity.
to each other. When pHLIP is at the surface of the vesicle and the pH is lowered, the protonation of one Asp residue might facilitate the protonation of other protonatable residues, shifting their pKₐ values. The protonation of the first Asp residue might induce partial insertion of the peptide into the membrane. In this scenario, the protonation of the neighboring Asp residues would be energetically favored to shield the negative charge (i.e., the pKₐ value of the neighboring Asp is shifted to higher values in a more hydrophobic environment) and then a positive feedback would be established, triggering membrane insertion.

How do the number and the location of Asp residues affect peptide exit from the membrane? The CD and fluorescence changes associated with wt pHLIP lipid insertion at acidic pH are completely reversible. Here we also followed changes in the CD and fluorescence signals and in the reversibility of biotin translocation across the membrane. The ellipticity increase associated with each peptide insertion into the membrane was found to be essentially reversible for wt and D3b (Fig. 3, broken blue lines overlap with continuous blue lines), while for D3a, D2, and D1, the reversibility was only partial. Since changes in the CD signal upon acidification for D2–D0 are less pronounced than those for wt and D3, the reversibility of the D2–D0 membrane insertion was also assessed by changes in the fluorescence signal (Fig. S4). It is interesting to note the different levels of reversibility of the two D3 peptides: the insertion process is significantly more reversible in D3b (90%) than in D3a (70%) (Fig. 5b).

---

Fig. 6. Fluorescence spectral maximum changes upon pH titration. The pH-controlled transitions of the peptides in POPC were followed by monitoring the variations in the spectral maxima. The experimental data for the different peptides were fitted to Eq. (1) (black lines). Representative experiments are shown.

Fig. 7. Parameters obtained from the fitting of fluorescence pH transitions. The pKₐ (a) and m parameter (b) values obtained from the fitting of the data in Figure 6 to Eq. (1) are shown in black symbols. Data from the D3b variant are shown as triangles (to maintain the representation as in Fig. 5). The line corresponds to the fitting of all data points (R² = 0.93). Averages and standard deviations are shown.
suggesting nonequivalence of the two buried positions. We observed an overall linear relationship between the number of aspartic acid residues interacting with the membrane and the degree of α-helix formation reversibility (Fig. 5b). The results obtained for the reversibility of the biotin translocation (exit process) were also in agreement (Fig. 5b).

An important consideration in the interpretation of the exit data is the time course of equilibration of the pH inside the liposomes, so we encapsulated the membrane-impermeable fluorescent probe 3(6)-carboxy-2',7'-dichlorofluorescein in POPC liposomes to follow the pH changes. The fluorescence of the probe is pH-sensitive, with a pK_a of 5.1. When we varied the pH of the solution outside the liposomes, the fluorescence of the encapsulated probe changed in a sigmoid fashion, with an apparent pK_a of 5.05 (data not shown). A relatively high proton permeation through unilamellar POPC liposomes in the minute timescale has been reported elsewhere. On the other hand, our kinetic data suggest that the time of wt peptide exit (with two TM groups and four C-terminal protonatable groups) is in the range of milliseconds. Thus, peptides exit from the lipid bilayer much faster such that the pH is completely equilibrated inside the liposomes and, most probably, C-terminal residues cross the membrane in their noncharged form. The question is: Why is the reversibility of D3a, D2, and, to some degree, D1 only partial? To provide an explanation, we take into account the location of the Asp residues. For the peptide exit from the lipid bilayer to take place, the deprotonation of Asp residues must energetically destabilize the inserted state. Destabilization of the inserted state is mainly caused by the charges resulting from the deprotonation of groups deeply buried in the hydrophobic core of the membrane. Therefore, the exit of wt and D3b, which have two Asp or one Asp in the hydrophobic core of the membrane, is fully reversible. The reason for the difference in peptide insertion reversibility between D3a and D3b might be related to the presence of an arginine residue at position 11. Accordingly, the deprotonation of Asp25 in D3b would strongly destabilize the membrane-inserted state due to the presence of a negative charge in the hydrophobic core of the membrane, favoring the exit process. However, the negative charge of Asp14 in D3a might be forming a salt bridge with the neighboring side chain of Arg11, which would result in a weaker destabilization of the inserted state. Another potential explanation is an altered position of the TM domain, which was mentioned above. There is a possibility that the TM domain in variants is shifted toward the C-terminal residues, leading to a greater exposure of the amino acid in position 14 (with His in D3a) to the aqueous environment and a shift to the hydrophobic core of amino acids at positions 31 and 33. As a result, the deprotonation of His14 in D3a might be associated with less destabilization of the helix than deprotonation of His25 in D3b. The side chains of Asp31 and Asp33 most probably are interacting with the headgroup region of the bilayer. The destabilization energy associated with their deprotonation is not enough to cause a complete exit from the membrane. Our results suggest that the deprotonation of acidic residues located in the hydrophobic core of the membrane ensures complete exit of the peptide.

**Discussion**

We have previously observed that even conservative changes in the pHLIP sequence can lead to peptide aggregation in solution at neutral pH. Our results show that all the peptides in this study are soluble in solution, being essentially monomeric (the addition of a D-tag at the N-terminus and the L26G mutation appear to favor peptide solubility). Spectral data obtained with D3–D0 peptides indicate that the lower is the number of negatively charged groups in the peptide sequence, the deeper are the peptide partitions into a lipid bilayer and the greater is the helicity. At the same time, TM orientation (at least for the D3–D2 peptides) requires protonation of the Asp/Glu residues and the terminal carboxyl group at the C-terminus, which can readily go across a membrane in its noncharged form. We confirmed our previous finding suggesting that TM Asp residues are not essential for peptide insertion. Interestingly, we have observed here that membrane insertion upon acidification occurs in our peptides in the presence of two His residues in the predicted TM region. Histidines have been used in the past to drive the insertion of peptides into membranes at neutral pH values. However, in these examples, acidic residues were completely absent in the sequence. For the peptides described in this article, the establishment of states II and III is driven by acidic residues. Since the protonated (charged) state of the side chains of His14 and His25 in the hydrophobic core of the membrane would be energetically very unfavorable, in the peptides, their pK_a values are expected to shift to lower values in the membrane-inserted state (favoring the unprotonated state). Further acidification eventually causes their protonation, resulting in a strong destabilization of the inserted TM helix and peptide exit. We cannot rule out that the diminished membrane insertion of the D1 and D0 peptides might be influenced by the hydrophobicity change concomitant to the Asp-to-Asn mutations at the C-terminus. The free energy of membrane transfer of the Asn side chain is 0.42 kcal/mol, which is a less favorable value than the free energy of transfer of the neutral state of Asp (−0.07 kcal/mol); thus, the
membrane translocation of the C-terminus would be less favorable. A similar effect might occur in the insertion reversibility of D1.

We conclude that protonation of negatively charged residues located in the TM or in the C-terminal inserting end must occur in order to preserve the pH-dependent ability of pHLIP to interact with the membrane. These residues act as switches for pHLIP membrane insertion, as the negative charges of their side chains block membrane insertion. Acidification causes the protonation of these side chains, resulting in an increase in the overall hydrophobicity of the peptide, which leads to TM helix formation, shielding the hydrophobic residues of pHLIP from water molecules. When the pH is raised to near neutrality, the negatively charged state of the carboxyl groups is again favored, decreasing the peptide hydrophobicity and resulting in exit from the TM position. Peptide exit from the lipid bilayer is completed when deprotonation of Asp/Glu residues located in the hydrophobic core of the membrane occurs and the TM helix is destabilized.

The knowledge gained from our experiments can be used as a guide to improve the imaging and therapeutic properties of pHLIP. For the specific case of tumor targeting, the pHLIP insertion characteristics should be finely tuned to exploit the low extracellular pH (pH_e) of tumors. Tumor targeting by wt pHLIP conjugated to a Cu\(^{61}\)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid chelate for positron emission tomography imaging correlates with the pH_e of tumors, where the contrast index was higher for LNCaP tumors (pH_e 6.78±0.29) than for PC-3 tumors (pH_e 7.23±0.10\(^{24}\)). Thus, pHLIP variants where Asp14/Asp25 were replaced by Glu, with a higher pH_e value, might be more effective for targeting tumors with higher pH_e values. Our present results suggest that the number of Asp residues in the TM region can also modulate the pH_e value. Thus, a peptide containing an extra Asp in the TM region might have a higher pH_e and might be directed to tumors more effectively. Another important factor to be considered is the broadness of the pH transition of the peptide, which is dictated by the cooperativity of the transition. On one hand, for the case where the peptide pH_e is lower than the tumor pH_e, but the transition is broad (in value is low), a significant part of the pH transition could intercept the pH_e value, resulting in a significant pHHLIP tumor insertion. However, such a scenario will also lead to more accumulation in healthy tissue. Since it is usually desirable to have a high tumor/organ ratio, an insertion transition of high cooperativity might be best. This would ensure greater differentiation between the amount of inserted peptides and the amount of noninserted peptides over a narrow range of pH values, favoring selective tumor targeting, since the difference in pH between normal tissue and cancerous tissue may be only 0.5–0.7 units. However, we must bear in mind that the measured pH_e provides an indication of the average acidity outside the cell for a given tumor and can vary between different tumor regions. Furthermore, pH_e may not reflect the precise pH on the exterior surface of the cells, since the cells pump protons to the extracellular medium and ΔpH will lead to proton accumulation at the membrane surface.\(^{29}\) Another feature that is expected to shift the equilibrium toward the membrane-inserted form is the presence of Asp/Glu residues at the C-terminus of the peptide. After being translocated across the plasma membrane into the cytoplasm, where the pH is neutral, these groups would be deprotonated. Since the translocation of charges across membranes is unfavorable, the inserted form would be stabilized.

pHLIP shows promise as a means of targeting cells in acidic tissues and delivering agents for therapy and imaging. At the same time, we are learning more about the binding and insertion of peptides at the membrane surface. Here we have shown that variation in the positions and numbers of carboxyl group titrations modulates the pK_a and cooperativity of insertion.

**Materials and Methods**

**Peptide synthesis and assessment of monomeric state**

Peptides were made by solid-phase synthesis, using standard 9-fluorenylmethyloxycarbonyl chemistry, at the W. M. Keck Foundation Biotechnology Resource at Yale University (New Haven, CT) and were purified by reverse-phase chromatography (C18 column, using a water/acetonitrile gradient in 0.01% trifluoroacetic acid). Purity was checked by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Peptides were quantified by absorbance spectroscopy using a molar extinction coefficient of 13,940 M\(^{-1}\) cm\(^{-1}\). Some peptides contain a single Cys residue in the C-terminus and thus have the potential to form intermolecular disulfide bonds, leading to the formation of dimers. To rule out the possibility that this might occur under our experimental conditions, we ran HPLC on peptide samples incubated (at room temperature for 3 h) at concentrations higher than those used in our experiments and in the absence and in the presence of POPC. No dimer band could be detected, and concentrations in the range of 0.1 mM peptide and overnight incubation were required to detect a significant amount of dimer (∼10%). The peptides described in Table 1 were used in the experiments, except for some experiments with D2–D0, where a Cys-less version was employed (similar results were obtained for both results; data not shown).
Analytical ultracentrifugation

Sedimentation velocity experiments were performed at 25 °C in a Beckman Optima XL-I analytical centrifuge at 35,000 rpm. Peptides at a concentration of 7 μM were dissolved in 5 mM phosphate buffer (pH 8) after 1 h of incubation at room temperature. Absorbance at 280 nm was used to monitor centrifugation, and analysis was performed using SEDFIT.26

Liposome preparation

The required amount of chloroform-dissolved POPC (Avanti Polar Lipids) was placed in a glass tube, dried with argon, and then held under vacuum overnight. The dried film was resuspended in water or 10 mM phosphate buffer (pH 8) and vortexed. Extrusion to make unilamellar vesicles was performed using a Mini-Extruder (Avanti Polar Lipids), with Nuclepore polycarbonate membranes of 0.1 or 0.05 μm pore size (Whatman). To obtain the final large unilamellar vesicles, we performed 15–25 extrusion steps, depending on the lipid concentration.

Fluorescence spectroscopy

Peptides were dissolved in 5 or 10 mM phosphate buffer (pH 8) and incubated with POPC vesicles prepared in water, resulting in a molar lipid/peptide ratio of 250:1. The incubation time with POPC liposomes varied from 90 min to 18 h. The pH of the samples was adjusted with 10 mM HCl. Addition of concentrated HCl. The final peptide concentration varied from 1.5 to 5 μM in different experiments. Emission spectra were measured in SLM-Aminco 8000C and PC2 ISS spectrophotometers at room temperature (controlled temperature), with excitation at 295 nm. The appropriate blanks were subtracted in all cases.

For determination of spectral maxima, we used the FCAT mode of the PFAST software, which fits the experimental spectra to log-normal components.27,28 The spectral maxima values for each point of the pH curve were plotted and analyzed according to29:

\[
F = \left( \frac{F_a + F_b 10^{m(pH-pK_a)}}{1 + 10^{m(pH-pK_b)}} \right)
\]

where \(F_a\) and \(F_b\) are the spectral maxima for the acidic and basic forms, respectively; \(S_a\) and \(S_b\) are the slopes of the acidic and basic baselines, respectively; and \(m\) is the cooperativity parameter. Fitting by nonlinear least squares analysis was carried out with Origin software.

Circular dichroism

Samples were prepared as in the fluorescence experiments, but the final molar lipid/peptide ratio was 300:1, with the final peptide concentration varying from 2 to 5 μM. CD spectra were recorded in Jasco J-810 and MOS450 Biologic spectropolarimeters interfaced with a Peltier system. Spectra were recorded at 25 °C using 2- or 5-mm cuvettes, the scan rate was 50 nm/min, and 10–30 averaging steps were performed. Raw data were converted into mean residue ellipticity according to30:

\[\theta = \Theta / (10lcn)\]

where Θ is the measured ellipticity, \(l\) is the path length of the cell, \(c\) is the protein concentration, and \(N\) is the number of amino acids.

For the study of membrane attachment, insertion, and its reversibility, the typical procedure was as follows: The samples were incubated with POPC vesicles at pH 8 for 90 min, the spectra were recorded, the pH was lowered to 4.0, and the measurements were performed after 30 min. Finally, the pH of the sample was increased with sodium borate buffer (pH 10.2) to a final pH of 7.5. After 30 min, 90 min, and 24 h, the spectra were recorded, and similar results were obtained in all cases. The degree of reversibility was established from the recovery of the signal at 222 nm. The final buffer concentration for the different experiments was in the range of 3–15 mM. Appropriate blanks were subtracted in all cases.

OCD measurements

For OCD measurements, supported bilayers were prepared on quartz slides with 0.2-mm-thick spacers on one side and with a special polish for far-UV measurements (Strata). Slides were cleaned by sonication for 10 min in cuvette cleaner solution (Decon Contrad 70, 5% in water), 2-propanol, acetone, and 2-propanol, and rinsed with deionized water. Then the slides were immersed in a mixture of concentrated sulfuric acid and hydrogen peroxide (3:1) for 5–10 min to completely remove any remaining organic material from the slides. The slides were then thoroughly rinsed with and stored in deionized water (Milli-Q purified water kept at 25 °C). A POPC lipid monolayer was deposited on a quartz substrate by the Langmuir–Blodgett method using a KSV mini-trough. For the Langmuir–Blodgett deposition, a cleaned slide was vertically immersed in the clean subphase (Milli-Q purified water kept at 25 °C) of a Langmuir–Blodgett trough. A POPC lipid solution in chloroform was spread on the subphase, and chloroform was allowed to evaporate for about 30 min, followed by monolayer compression to 32 mN/m. The first layer was deposited by retrieving the slide from the subphase at a rate of 15 mm/min. The second layer of the bilayer was created by fusion. For this step, the monolayer on the slide was incubated with a solution of POPC vesicles (50 nm in diameter, obtained by extrusion) mixed with peptide solution at the required pH (0.5 mM POPC and 10 μM peptide). The fusion occurred for about 6 h under 100% humidity. Then, excess vesicles were carefully removed, and the slides were stacked to make a pile while filling up the spaces between them with a peptide solution (5 μM) at the required pH. The bilayers with the peptide solution were allowed to equilibrate for about 6 h. Measurements were taken in three steps during the process: when the monolayers were incubated with an excess of liposomes, soon after the spaces between the bilayers had been filled with the peptide solution, and 6 h after the second measurement. Fourteen slides (28 bilayers) were assembled, and the
OCD spectrum was recorded on a MOS-450 spectrometer at a sampling time of 2 s.

**Biotin translocation assay**

HABA dye (4′-hydroxyazobenzene-2-carboxylic acid) binds to avidin at a 1:1 stoichiometry and absorbs at 510 nm only in the avidin-bound state. This interaction is strongly displaced by the binding of biotin to avidin, resulting in a quantitative reduction in HABA absorbance. This property was used to probe the location of the C-terminus of different peptides with regard to the liposome (inside or outside) (method modified from Nicoll et al.31). The C-terminus of each of the peptide variants was labeled with biotin (see the text below). The rationale for the assay is that pH-driven insertion of the C-terminus would result in biotin translocation inside the liposome, causing shielding of the biotin from the medium outside the liposome, where a preformed HABA/avidin complex (Thermo Scientific) is added. If the biotin is inside the liposome, no change in absorbance is expected. On the other hand, if pHLIP lies at the exterior surface of the liposome, the C-terminal biotin would be accessible to the solution outside the liposome (as the biotin group is polar, it is expected not to be protected by the membrane) and would be able to bind to avidin and displace the HABA/avidin complex, with a consequent reduction in absorbance at 510 nm. Liposomes were prepared in 150 mM NaCl, and ionic strength was carefully maintained during all steps to avoid liposome osmotic shock. Biotin-labeled peptides were incubated in the presence of POPC at pH 8 for 2 h at room temperature (150:1 lipid/peptide ratio). For studies of C-terminal translocation, acetate buffer was added to the samples, resulting in a final pH of 4.3 prior to 1 h of incubation with the peptide. The HABA/avidin complex was added to the solution only after the final conditions had been established. The final peptide concentration for the measurement conditions was 3 μM. To determine the reversibility of the biotin translocation, we increased the pH by the addition of 10 mM sodium borate buffer (pH 10.2) to give a final pH of 7.4. Absorbance was measured after 1 h of incubation. For quantitation of the level of reversibility, the recovery of absorbance obtained for pHLIP labeled with biotin at its C-terminus was taken as 100% reversibility, and that of pHLIP labeled at its N-terminus was taken as 0%.

Peptides were labeled at the C-terminal Cys residues using the membrane-impermeable compound maleimide–PEG–biotin (Thermo Scientific), which has a long polar spacer arm of 29.1 Å to allow adequate biotin binding to avidin. The synthesis reaction was performed in 10 mM phosphate buffer (pH 7.5; overnight incubation at 4 °C). Reaction products were purified by HPLC, and the mass of the biotin-labeled peptides was checked by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The octanol/water partition coefficient of maleimide–PEG–biotin was determined experimentally by measuring the absorbance at 300 nm in the aqueous and octanol (previously pre-equilibrated with water) phases after 2 h of vortexing. A logP value of −1.07 ± 0.02 was obtained. As this value does not take into account the chemical changes in the cross-linking reaction (formation of a thioether bond between the maleimide moiety and the Cys side chain), the QikProp 3.0 software was employed to predict the logP value of the reacted form, resulting in a value of −1.4, which is in the range of molecules that can be translocated by pHLIP.21

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.08.010

**Acknowledgements**

The authors are thankful to Miriam Alonso, Ming An, Justin Fendos (Yale University), and José Luis Neira (Universidad Miguel Hernández) for stimulating discussions and insightful comments on the manuscript. D.M.E. was supported by National Institutes of Health grant GM073857-04. O.A.A., D.M.E., and Y.K.R. were supported by National Institutes of Health grant CA133890-03. F.N.B. was the recipient of a postdoctoral fellowship from the Fundación Alfonso Martín Escudero.

**References**