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pH (low) insertion peptide (pHLIP) inserts across a lipid bilayer as a helix and exits by a different path

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What are the molecular events that occur when a peptide inserts across a membrane or exits from it? Using the pH-triggered insertion of the pH low insertion peptide to enable kinetic analysis, we show that insertion occurs in several steps, with rapid (0.1 sec) interfacial helix formation, followed by a much slower (100 sec) insertion pathway to give a transmembrane helix. The reverse process of unfolding and peptide exit from the bilayer core, which can be induced by a rapid rise of the pH from acidic to basic, proceeds approximately 400 times faster than folding/insertion and through different intermediate states. In the exit pathway, the helix-coil transition is initiated while the polypeptide is still inside the membrane. The peptide starts to exit when about 30% of the helix is unfolded, and continues a rapid exit as it unfolds inside the membrane. These insights may guide understanding of membrane protein folding/unfolding and the design of medically useful peptides for imaging and drug delivery.

folding kinetics | helix formation | lipid-peptide interaction | membranes | transmembrane helix

The stability and folding of membrane proteins are strongly constrained by the formation of secondary structures in the lipid bilayer environment, driven by the hydrophobic effect and hydrogen bonding. Consideration of these factors has led to versions of a thermodynamic framework model for the folding and unfolding of helical membrane proteins (1–5). One concept is that spontaneous insertion and folding includes the formation of helical intermediates at the bilayer surface, followed by insertion, and that unfolding includes the same steps, but in reverse order. Because folding to form a helix is coupled to insertion, a significant experimental challenge in testing the concepts is to separate the process of peptide partitioning into a membrane from the folding events leading to secondary structure.

The pHLIP (pH low insertion peptide) gives an opportunity to observe membrane-associated transitions between surface coil and transmembrane helix and vice versa. At neutral and high pHs pHLIP is monomeric at concentrations <8–10 μ M, and equilibrates between unstructured forms in aqueous solution (state I) and bound to the surface of a lipid bilayer if one is available (state II) (6, 7) (Fig. 1A). In an acidic environment the equilibrium is shifted toward a monomeric transmembrane helical form (state III) (6, 7), and the process of insertion is accompanied by an energy release of about 1.8 kcal/mol in addition to the binding energy (6–7 kcal/mol) locating the peptide at the surface (8). The pK_a of the transition from state II to state III is 6.0 (6, 8). The insertion is driven by the protonation of two Asp residues in the transmembrane region, leading to an increase of hydrophobicity that results in the folding of the peptide across a membrane (9). Increasing the pH promotes the unfolding and exit of the peptide from the core of the lipid bilayer. The insertion of pHLIP across a membrane is unidirectional: The C-terminus goes inside a cell or vesicle, and the N-terminus stays outside (7, 10). Neither partitioning of an unstructured peptide onto the bilayer surface at neutral pH, nor insertion as a transmembrane helix at low pH

promotes membrane fusion nor leakage of vesicles, red blood cells, or cancer cells (7, 9, 10).

Because pH changes can be accomplished by rapid mixing, it is possible to study the kinetics of the insertion and exit processes, and to examine the sequences of events that are involved. Here we present the results of such a kinetic investigation of pHLIP insertion/folding and exit/unfolding, which lead to new insights on the pathways.

Results

Steady-state fluorescence and CD spectroscopy are useful ways to monitor conformational and environmental changes as pHLIP experiences transitions between its three principal states (Fig. 1B and C). Spectroscopic signals in solution provide a good assessment of the peptide helicity as a function of pH and in the absence and presence of the lipid bilayer, but they do not allow a distinction between membrane-surface and transmembrane orientation of the helical form. We previously used FTIR to make this distinction, and now add to that evidence using oriented circular dichroism (OCD) measurements. It has been predicted and confirmed experimentally (11) that, for planar samples with the incident light perpendicular to the planes of the bilayers, a membrane-surface orientation of the helix gives a CD signal similar to the α -helical CD signal in solution (similar to the red line in Fig. 1C). However, if the helix axis has a transmembrane orientation, the incident light is parallel to the axis and several transitions are degenerate giving a characteristic CD spectrum with a positive amplitude around 200 nm and a shift of the negative amplitude to longer wavelengths (225–230 nm) (Fig. 1D) (for more information about OCD spectroscopy see (11) and references therein). CD spectroscopy on oriented supported bilayers has been successfully applied to distinguish membrane-surface and transmembrane helix orientation (12). We obtained a characteristic OCD spectrum for transmembrane helix orientation using direct insertion of pHLIP peptides into supported bilayers at low pH (in contrast to earlier experiments, where bilayers were assembled with membrane peptides). The results are in excellent agreement with our previous FTIR data demonstrating transmembrane helical orientation of the pHLIP at low pH (6), and with a number of studies indicating that the C-terminus of pHLIP inserts across the lipid bilayer (7, 10). Transmembrane helix formation is accompanied by the appearance of a characteristic helical CD signal, by a shift of the fluorescence spectrum maximum and by an increase of fluorescence

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The authors declare no conflict of interest.

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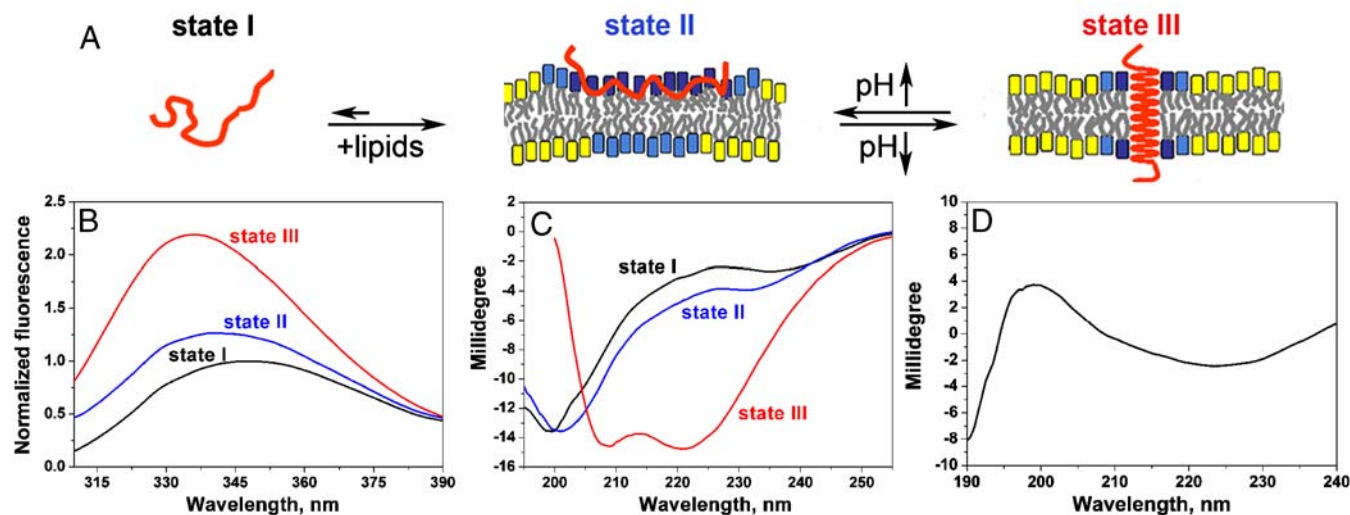


Fig. 1. (A) A schematic representation of pHLIP in solution and interacting with a lipid bilayer at neutral and low pHs is shown. State I refers to the peptide in solution at normal and basic pHs. Upon addition of vesicles at pH 8, the unstructured peptide is adsorbed on the membrane surface (State II). A drop of pH leads to the protonation of Asp residues, increasing peptide hydrophobicity, and resulting in the insertion and formation of a transmembrane α -helix (State III). Lipids interacting with the peptide directly are marked with blue head groups, lipids influenced by the interaction but not interacting with the peptide directly have yellow head groups, and lipids that are not involved in the interaction with pHLIP have yellow head groups (8). Transitions between states can be monitored by (B) changes of fluorescence and (C) circular dichroism (CD) spectral signals. The fluorescence and CD spectra of pHLIP at pH8 (Black Lines) indicate an unstructured configuration with tryptophan residues fully exposed to solvent. Incubation of pHLIP with liposomes at pH8 (Blue Lines) induces the partial burial of tryptophan residues inside the lipid bilayer without helix formation. Decreasing the pH to 4.0 by the addition of HCl (Red Lines) induces the insertion of pHLIP and helix formation. (D) The transmembrane orientation of the helix has been confirmed by OCD (see text and ref. 11 for discussions of OCD spectroscopy).

intensity (Fig. 1B and C). These signals are the basis of our kinetic studies.

An advantage of our system is that the initial states in the process of folding/entry or unfolding/exit are well defined. For the folding/insertion study, the initial state is the peptide bound to the surface of a membrane as an unstructured monomer; for the unfolding/exit experiments the peptide starts as a transmembrane helix. The choice of experimental conditions (peptide: POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine) molar ratio at approximately 1:140) is based on the results of our previous studies of peptide interaction with POPC vesicles at different temperatures (8), and is chosen to avoid crowding of the peptide on the surface of vesicles (the “parking problem”). Given the high pH surface binding energy of 6–7 kcal/mol observed for temperatures in the range from 15 to 37 °C (8), the bound to free ratio is approximately 10^4 – 10^5 , so the initial state in the insertion experiments at all temperatures is predominantly an unstructured surface bound state of the peptide. In the unfolding experiments, the initial state, at low pH, is predominantly the transmembrane helical configuration of the peptide (>95% state III).

Transitions between states can be induced by rapidly changing pH using fast mixing of a aqueous solutions of pHLIP preincubated with POPC at pH 8.0 or pH 4.0 with diluted solutions of HCl or NaOH, respectively. Membrane-associated peptide folding/insertion and unfolding/exit were monitored by changes of CD and fluorescence signals (Fig. 2). For fast fluorescence measurements, a filter was used to capture the emission, but changes of the entire fluorescence spectrum were also recorded in a global mode with use of an emission monochromator (Fig. 2C and F). The fluorescence spectra obtained in stopped-flow mode clearly show that the increase of fluorescence is accompanied by a shift of the maximum, which indicates peptide insertion into the hydrophobic core of the membrane.

We find that the process of helix formation, triggered by a pH drop, occurs on the bilayer surface within 1 sec (about 90% of total changes of CD signal), whereas insertion of the helix across the membrane takes approximately an additional 100 sec

(Fig. 2A–C). Intriguing results were also obtained for the unfolding/exit pathway, which proceeds much faster than folding/insertion (Fig. 2D). Starting with the inserted helix, a rapid increase of pH leads to a short delay (about 12 msec), followed by a decrease of about 90% of the fluorescence signal (Fig. 2E) and the helix–coil transition, all within the next 0.3 sec, followed by the final equilibration of the peptide at the surface of the lipid bilayer until the signal reaches a stable level (10–20 sec). To estimate activation energy parameters for the process of peptide insertion into the lipid bilayer, we recorded the kinetics curves at five different temperatures (Fig. 3A). Increasing the temperature speeds up the process of insertion, presumably as a result of increased thermal fluctuations. Steady-state fluorescence spectroscopy was used to measure the total changes of the fluorescence signal at various temperatures as a result of the transition from states II to III and vice versa. The total fluorescence changes were relatively unchanged over a range from 11 to 37 °C, allowing us to conclude that the process of peptide insertion is completed to the same extent at all temperatures in our study.

Our experimental data clearly show differences between the pHLIP folding and insertion and unfolding/exit pathways. To gain further insights, we fit the data with a sequential kinetic model. In general, parallel pathways may exist in heterogeneous systems, where peptide isomers or oligomers may present, so we selected initial experimental conditions where pHLIP is monomeric and predominantly bound to the bilayer surface in an unstructured configuration or inserted as a helix across the lipid bilayer (7, 8). Another potential source of heterogeneity might be associated with isomerization of the Pro located in the middle of the transmembrane part of the peptide. To examine this possibility, a pHLIP variant was synthesized with Pro replaced by Ala. Unfortunately, the variant shows some helical structure in solution at neutral pH (Fig. S1), and binding of the variant to a vesicle at high pH further promotes the coil–helix transition. Because replacement of Pro led to conformational changes of the peptide in solution and in peptide–bilayer interactions, we asked whether the presence of prolyl isomerase (cyclophilin A), which promotes

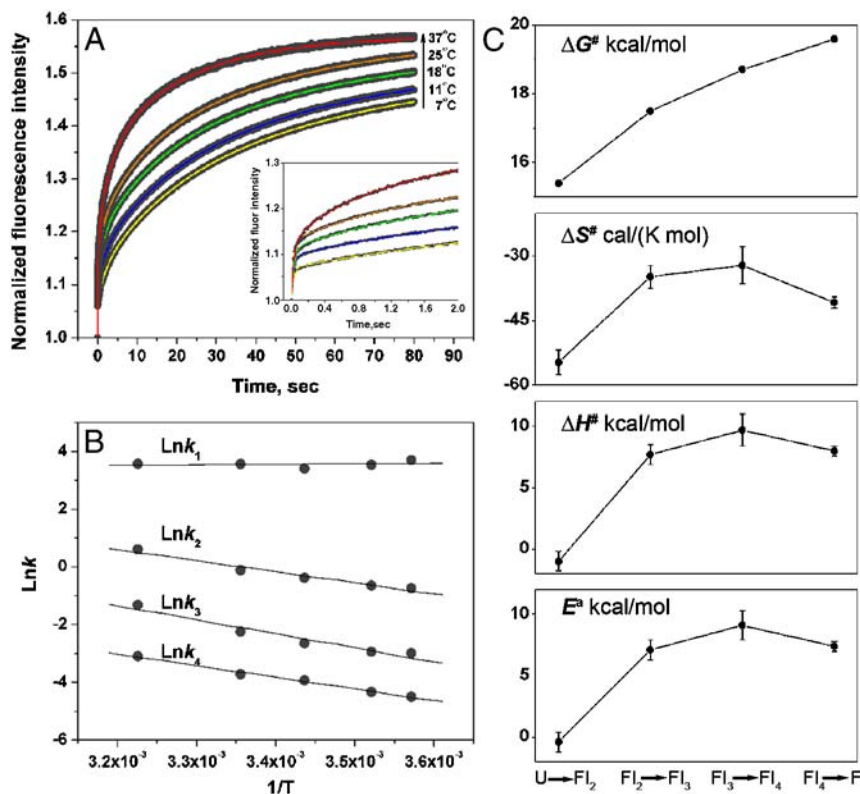


Fig. 3. (A) The membrane-associated pHLIP insertion across the lipid bilayer of POPC vesicles was monitored by changes of the fluorescence signal through a 320 nm cutoff filter at an excitation wavelength of 280 nm and at various temperatures. The fitting curves obtained at various temperatures are color coded. The data were fitted by the kinetic model with three intermediates by using function (Eq. S3). (B) The Arrhenius plot was constructed according to the Arrhenius equation. (C) The changes of the activation energy, E^a , enthalpy ΔH^\ddagger , entropy ΔS^\ddagger and Gibbs free energy, ΔG^\ddagger for each transition are plotted. The values of the thermodynamic activation parameters are presented in Table S2.

and unfolding/exit from the bilayer, and we used them to follow the pathways in real time.

Earlier pH-jump experiments monitored by changes of pHLIP fluorescence were reported by Hunt et al. in 1997 (6), but these did not include measures of secondary structure. More recently, a fluorescence kinetic study was reported by Tang and Gai (14), who followed peptide binding to the surface and insertion into a bilayer. As in the Hunt study, the CD signal was not measured and only the intensity of tryptophan fluorescence was recorded. Moreover, the kinetic process was monitored for only the first 5 sec out of 100 sec, so >50% of the fluorescence signal changes were missed. We have expanded on these earlier efforts using stopped-flow measurements of both the fluorescence parameters (intensity and wavelength position of maxima of spectra) and the CD signal. The results clearly demonstrate differences in the processes of (i) coil-helix transition and peptide insertion into membrane, and (ii) peptide folding/entry and unfolding/exit from the membrane. Our main findings are that the entry pathway begins with rapid helix formation, followed by slow transbilayer insertion, and that the exit pathway is distinctly different.

In an attempt to gain more insights on the folding and unfolding pathways we introduced a kinetic model to fit the experimental curves, resulting in the mechanistic model shown in Fig. 4. Some features of the model are viewed with confidence whereas others are much less certain, and we can only formulate educated guesses as to the nature of several intermediate states. An important caveat is that the model assumes single pathways of insertion and exit. Starting with the surface bound peptide of State II, and following the drop in pH, we see a rapid formation of helix, in two steps (folding intermediates 1 and 2, forming at 8 msec and 112 msec), each producing about half of the total helix. The helix might be a single straight helix, or it might consist of several short

helices with breaks in between (these possibilities are experimentally indistinguishable). This part of the interpretation is secure: Regardless of the kinetic model, helix formation clearly is faster than most of the fluorescence changes. It is possible that slight sinking of a polypeptide during helix formation or helix rotation at the interface account for the fast first (23%) fluorescence change. Following the rapid helix formation, the rest of the insertion process is reported by fluorescence, and is about 1,500 times slower, describable as several kinetically distinct steps over approximately the next 100 sec.

We cannot be certain of the exact nature of the kinetic intermediates during insertion, but we suggest a few thoughts as working ideas. From the surface helix formation to the fully inserted transmembrane helix, two intermediate states (FI_3 and FI_4) followed from our analysis, and are the rate limiting steps in the insertion process. We assume that at least one of these intermediates is related to the translocation of the polar C-terminus of pHLIP across the bilayer. It may be that the fourth intermediate is a transbilayer inserted form with the C-terminus across the bilayer, or that the C-terminus crosses the bilayer during the last transition. We have no direct evidence yet to choose one of these, but we know that the C-terminus must cross the bilayer at some point. Each step from the unfolded to the folded state is associated with a decrease of entropy (decrease of disorder) that reflects the process of folding (ordering). It is possible that both the peptide and the lipids undergo a disorder-order transition.

In contrast with previously proposed theoretical models suggesting that entry and exit pathways mirror each other, our data reveal that the unfolding and exit of the peptide in response to a sudden pH increase occurs within 100 msec, orders of magnitude faster than insertion. Assuming that the increase of pH leads to the deprotonation of the Asp residues [perhaps involving proton

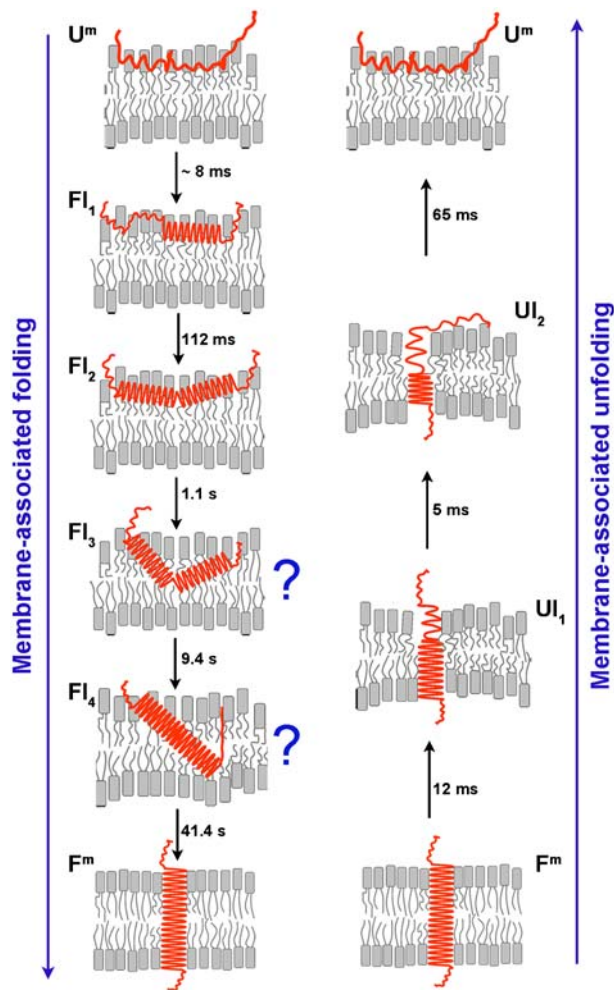


Fig. 4. A schematic interpretation of the membrane-associated folding and unfolding events suggested by the kinetic data. The pHLIP starts as a surface-bound coil at pH8. A rapid drop of pH to 4 causes a rapid formation of helical structure on the surface, followed by a much slower insertion of the helix across the bilayer, with several kinetically distinct intermediates for which speculative models are shown. A sudden elevation of the pH from 4 to 8 results in the rapid unfolding and exit of the peptide via a different pathway with different intermediates (see discussion in the text).

transfer from Asp86 to Arg83, which is known to happen in bacteriorhodopsin, from which the pHLIP is derived (15, 16)], followed by the helix-coil transition and a rapid exit of the polypeptide from the bilayer interior. Surprisingly, about 30% of the helix-coil transition is completed within approximately 12 msec, while practically no change of the fluorescence signal is observed, indicating that the polypeptide remains in the membrane interior as an unfolding intermediate (UI_1). Thus, partial unfolding of the TM helix, involving about 5–6 residues out of 20–22 residues, might occur inside the membrane and the peptide subsequently moves out of the bilayer interior. Another possibility is unfolding of part of the helix, followed by immediate exit of the polypeptide from the membrane, which leads to a thinning of the lipid bilayer around the shortened helical part of the peptide. This suggestion is based on recent findings that the lipid bilayer can accommodate short helices with a minimal energetic cost (17, 18). Further propagation of the helix-coil transition is accompanied by a rapid exit of part of the polypeptide within the next 5 msec (UI_2). The remaining 30% of the membrane-embedded helical structure unfolds and exits within 65 msec (including the C-terminus), and equilibrium is established between pHLIP in its unfolded

membrane-bound (U^m) and soluble forms within the final 10–20 sec. Unfolding experiments carried out at 7°C showed that the peptide exit also occurs in two steps with characteristic times of 17.6 and 152 msec. Because the C-terminus, which must cross the bilayer, has several carboxyl groups, an interesting possibility is that protonation and deprotonation steps might account for steps in the entry and exit pathways.

Several authors have reported peptide coil-helix transitions induced by binding to bilayers and the existence of interfacial folded intermediates, mostly using fluorescence approaches (19–24). Although interesting, the results of molecular dynamics simulations of membrane-associated folding and insertion of various peptides are not definitive (25–28). The pHLIP presents a case where the binding of the pHLIP to the membrane itself does not promote folding, so a stable, unstructured state can be established at a bilayer surface. The protonation of charged Asp residues, which leads to increased hydrophobicity of the peptide, induces a coil-helix transition and peptide insertion. We show that the formation of an interfacial helical intermediate is a step in the pathway of pHLIP insertion into a membrane. Helix formation reduces the free-energy penalty associated with the partition of the peptide backbone into the low dielectric environment of the bilayer, despite the fact that the coil-helix transition is associated with a loss of entropy. Helix insertion, most probably, is accompanied by a significant perturbation of lipids beyond that of the surface bound state, although the lipid perturbation is reduced in the overall process. The insertion of pHLIP is slow, because the peptide is initially located on the outer leaflet of the bilayer, and it takes time for the polar C-terminus to cross the membrane and to reorganize lipids around the transmembrane helix. In contrast to the insertion, unfolding and exit occur much faster, perhaps because the peptide can be conceptualized as occupying a small channel across the lipid, so that the peptide can quickly exit without the significant lipid reorganization needed for the intermediate states of insertion. Such a channel would close immediately after exit of the peptide.

We have demonstrated *in vivo* that pHLIP can target diseased tissues with elevated levels of extracellular acidity, such as tumors (9, 29, 30) and that the energy of the insertion events can be used for the selective translocation of polar cell-impermeable cargo molecules across the membranes of liposomes and cells (10, 30). Our kinetic data provide insights on the mechanisms of membrane-associated polypeptide folding and unfolding, and may be useful in improving the design of peptide based transmembrane delivery agents.

Methods

pHLIP Peptide. The pHLIP sequences AEQNPIYWARYADWLFITPLLLDLALLV-DADEGT and its Pro to Ala variant AEQNPIYWARYADWLFITALLLDLALLV-DADEGT were prepared by solid-phase peptide synthesis using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry and purified by reverse phase chromatography at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. For use of the peptide, the lyophilized powder is dissolved in a solution containing 3 M urea and transferred to 10 mM phosphate buffer, pH8.0 using a G-10 size-exclusion spin column. The concentration of the peptide was determined by absorbance ($\epsilon_{280} = 13,940 \text{ M}^{-1} \text{ cm}^{-1}$).

Vesicle Preparations. Large unilamellar vesicles were prepared by extrusion. 1 ml of 25 mg POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids, Inc.) in chloroform was desolvated on a rotary evaporator and dried under vacuum for several hours. The phospholipid film was rehydrated in 10 mM phosphate buffer, pH 8.0, vortexed for 2 h, and repeatedly extruded using a 50 nm membrane pore size.

Steady-State Measurements. Steady-state fluorescence measurements were carried out on a PC1 spectrofluorometer (ISS, Inc.) under temperature control. Peptide fluorescence spectra were recorded from 310 nm to 400 nm with the spectral widths of excitation and emission slits set at 2–4 nm and 2 nm, respectively, using excitation wavelengths of 275, 280, and 295 nm. Changes of the fluorescence signal in the process of transition from one state to

another are independent of the excitation wavelength, indicating that the spectral contribution of Tyr fluorescence is minimal. The polarizers in the excitation and emission paths were set at the "magic" angle (54.7° from the vertical orientation) and vertically (0°), respectively, to reduce Wood's anomalies from the reflecting holographic grating. Steady-state CD and OCD measurements were carried out on a MOS-450 spectrometer (BioLogic, Inc.) under temperature control. All measurements were performed at 25 °C unless otherwise indicated. The detailed description of the OCD measurements including preparation of samples can be found in *SI Text*.

Stopped-Flow Measurements. Stopped-flow fluorescence and CD measurements were carried out on a SFM-300 mixing apparatus connected to a MOS-450 spectrometer (BioLogic, Inc.) under temperature control. The FC-20 and TS-100/15 observation cuvettes were used for the fluorescence and CD measurements, respectively. All solutions were degassed several minutes under vacuum before loading into the syringes to minimize air bubbles. pHLIP (7 μM) was preincubated with POPC (1 mM) at pH8.0 to reach binding equilibrium and folding/insertion was induced by fast mixing (5.7 ms dead time) of equal volumes of pHLIP-POPC pH8 and appropriately diluted HCl, to obtain a drop of pH from 8 to 4. In the unfolding experiments, pHLIP was preincubated with POPC at pH8.0, then HCl was added to lower the pH to 4.0, and time was allowed for equilibration (minutes). Unfolding

was induced by mixing equal volumes of pHLIP-POPC pH4 and NaOH diluted to increase the pH from 4 to 8. In majority of cases, samples were collected after the shots and steady-state fluorescence were recorded on a PC1 spectrofluorometer. In the case of experiments in the presence of prolyl isomerase (cyclophilin A), which catalyses the isomerization of Pro residues in peptides, pHLIP was preincubated with the cyclophilin A (Sigma) at a ratio of 1:100 for 2 h before mixing with liposomes, followed by fluorescence (at various temperatures) and CD kinetics measurements. Additional information on the stopped-flow measurements can be found in *SI Text*.

Data Analysis. The kinetic equations were solved by integration (Eq. S1) in Mathematica 5 (Wolfram Research). Nonlinear least squares curve fitting procedures were carried out in Origin 7 and MatLab. The goodness-of-fit was assessed by the adjusted R-square statistics ($\text{adj}R^2$) and rms error according to the standard formula. The details of the kinetic model used in the study and description of the calculated thermodynamic activation parameters can be found in *SI Text*.

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