



Aggregation Behavior of pHLIP in Aqueous Solution at Low Concentrations: A Fluorescence Study

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Abstract

pH (low) insertion peptide (pHLIP) is a 36-residue peptide derived from the third transmembrane helix of the membrane protein bacteriorhodopsin. The hydrophobicity of this peptide makes it prone to aggregation even at low concentrations, but this has not been studied in detail. In this work, we characterized monomeric and aggregated forms of pHLIP in aqueous solution (pH 8) at low concentrations ($\sim\mu\text{M}$) using fluorescence-based approaches, complemented by circular dichroism (CD) spectroscopy. We show here that monomeric and aggregated pHLIP display differential red edge excitation shift (REES) and CD spectra. These spectroscopic features allowed us to show that pHLIP aggregates even at low concentrations. A detailed knowledge of the aggregation behavior of pHLIP under these conditions will be useful for monitoring and quantifying its interaction with membranes.

Keywords pHLIP · Peptide aggregation · REES · Low concentration · CD spectroscopy

Introduction

pH (low) insertion peptide (pHLIP) is derived from the third transmembrane helix of the integral membrane protein bacteriorhodopsin [1], which is the major membrane protein found in halobacteria such as *Halobacterium halobium*. It is a small polypeptide containing 36 amino acid residues with the sequence GGEQNPIYWARYADWLFTPLLLDLALLVDADEGT (see Fig. 1a) [2]. The peptide is predominantly composed of hydrophobic and polar residues, interspersed with few charged residues, mainly aspartate and glutamate, with an overall charge of -5 at physiological pH. The amino acid sequence of pHLIP contributes to its amphipathic nature, which is a characteristic

feature of peptides that interact with membranes. pHLIP is intrinsically fluorescent due to the presence of two tryptophan residues, which allows monitoring of its conformation and dynamics by fluorescence spectroscopy-based approaches.

An important characteristic of pHLIP is that it exists in different conformations depending on peptide concentration, pH and its environment. pHLIP exists mostly in unstructured random coil conformation at pH 8 in aqueous solution, and upon interaction with membranes, gets adsorbed to them [2]. Upon lowering pH to ~ 4 , the peptide spontaneously inserts into the membrane and adopts an α -helical conformation where the C-terminus traverses the bilayer, while the N-terminus stays on the *cis* side (outside the membrane). Previous work has shown that insertion of pHLIP takes place as a result of protonation of two aspartate residues (located in the transmembrane region) at low pH, which causes an increase in peptide hydrophobicity and triggers insertion into the lipid bilayer [3]. The conformational plasticity displayed by pHLIP makes it a promising candidate for studying protein folding and insertion into membranes. Consequently, the binding of pHLIP to membranes has been studied utilizing a number of techniques [4, 5]. The pH-dependent conformational switch has been utilized to translocate cargo molecules across the lipid bilayer on lowering pH [6–8]. This property of pHLIP has been successfully utilized in cancer biology since low (acidic) extracellular pH is a hallmark observed in tumor

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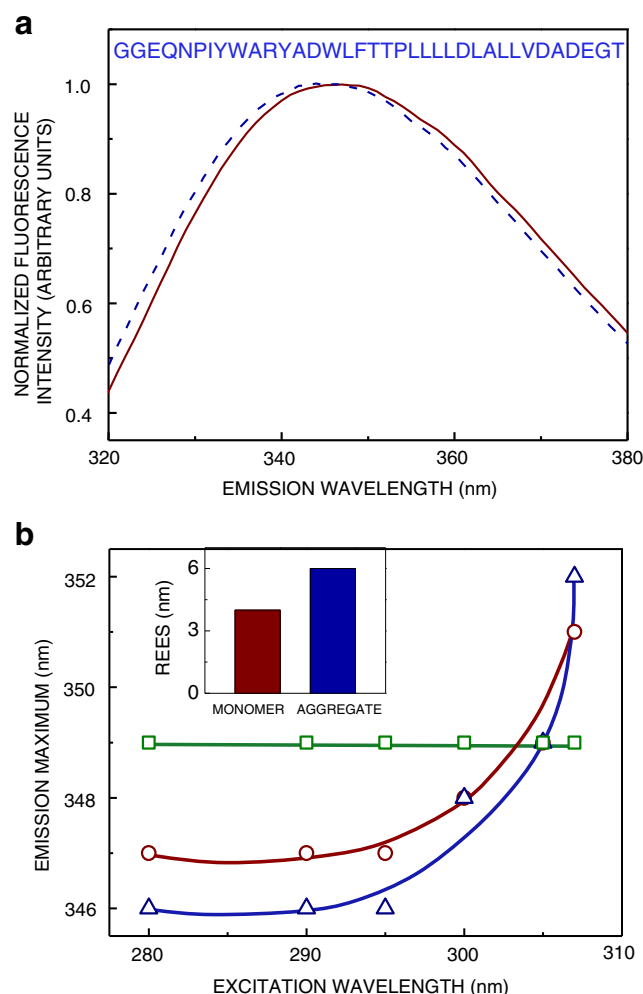


Fig. 1. (a) Representative tryptophan fluorescence emission spectra of monomeric (—) and aggregated (---) pHLIP in 10 mM phosphate buffer at pH 8. The fluorescence spectra were intensity-normalized at the emission maximum. The amino acid sequence of pHLIP is shown at the top. (b) Effect of altering the excitation wavelength on the wavelength of emission maximum for monomeric (○), aggregated (Δ) and denatured (□) pHLIP. Inset shows the magnitude of REES, which is equivalent to the shift in emission maximum when the excitation wavelength was shifted from 280 to 307 nm, for monomeric (left bar) and aggregated (right bar) pHLIP. Data shown are representative of at least three independent measurements and the observed emission maxima were identical or within ± 1 nm of the values reported. The concentration corresponding to monomeric and denatured pHLIP was 3 μ M, and that of aggregated pHLIP was 12 μ M. Lines joining the data points serve only as viewing guides. See Experimental for further details

tissues [9–12]. In particular, it has been shown that pHLIP localizes at tumor sites in mice and rats at low pH [9].

pHLIP exists in a random coil conformation at low peptide concentrations at pH 8. Upon increasing its concentration, pHLIP associates to form α -helical oligomeric structures with circular dichroism (CD) spectral signature characteristic of an exciton peak at very high ($\sim$ mM) concentrations [2]. The degree of aggregation varies depending on factors such as ionic strength and temperature [13]. The process of aggregation is

reversible as aggregates are converted into monomers upon dilution, but dissociation of the peptide aggregates is believed to be a slow process. Importantly, the aggregation propensity of pHLIP could influence its interactions with membranes, but has not been studied at low ($\sim$ μ M) peptide concentrations, particularly using spectroscopic (fluorescence and CD) approaches.

Fluorescence-based approaches are useful in understanding the properties of amphipathic peptides which are prone to aggregation, since fluorescence is often sensitive to aggregation, even at low concentrations [14]. We previously monitored the aggregation behavior of the hemolytic peptide melittin [15] in solution upon varying the ionic strength [16]. In this work, we utilized fluorescence-based approaches to characterize the monomeric and aggregated forms of pHLIP in aqueous solution at low concentrations ($\sim$ μ M range). To this end, we used steady state and time-resolved fluorescence spectroscopy measurements, including red edge excitation shift (REES), along with CD spectroscopy, to monitor the monomeric and aggregated forms of pHLIP. Our results show that the aggregated form of pHLIP shows more restricted solvent dynamics in the microenvironment of the tryptophan residues, as reported by REES measurements, and exhibit marked difference in CD spectra. These results are indicative of pHLIP aggregation, even at relatively low concentrations in solution, and will be useful for monitoring the interaction of pHLIP with membranes.

Experimental

Materials

Na_2HPO_4 , NaH_2PO_4 , urea (BioUltra grade) and dialysis kit with 1 kDa cut-off were purchased from Sigma Chemical Co. (St. Louis, MO). NaF was purchased from Sisco Research Laboratories Ltd. (Mumbai, India). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Peptide Synthesis and Purification

pHLIP was synthesized by solid-phase peptide synthesis [13]. The peptide was synthesized in a 433 A Peptide Biosynthesizer from Applied Biosystems (Foster City, CA) using Fmoc chemistry. Purification was performed on a LC-8A liquid chromatography instrument from Shimadzu (Duisberg, Germany) equipped with a ProntoSil C18 PREP2025 column (Leonberg, Germany). The identity and purity of the peptide were measured with a Waters Acquity UPLC, equipped with an Atlantis dC18 (30×2.1 mm, 3 μ m) column, coupled to a LCT premier TOF Mass Spectrometer from Waters (Guyancourt, France). The purity of the peptide was $>97\%$. The peptide was stored in lyophilized aliquots at -20 $^\circ\text{C}$ till further use.

Sample Preparation

As pHLIP is hydrophobic and prone to aggregation, it was dissolved in 10 mM phosphate buffer (pH 8) containing 6 M urea at room temperature (~23 °C) by occasional vortexing for 10 min. This was followed by dialysis of the peptide at 4 °C using dialysis kit against 100 mM phosphate buffer (pH 8) containing 10 mM NaF for 24 h with three changes of buffer. The dialysis membrane was equilibrated in the same buffer for 15 min prior to use. The concentration of pHLIP was estimated from its molar extinction coefficient (ϵ) of 13,940 M⁻¹ cm⁻¹ at 280 nm [2]. Stock solutions of the peptide were stored at 4 °C. Peptide solutions of different concentrations were prepared by adding the peptide from a concentrated stock solution into the buffer. Final peptide solutions were incubated overnight to equilibrate before fluorescence and CD measurements.

Steady State Fluorescence Measurements

Steady state fluorescence measurements were carried out using a Fluorolog-3 Model FL3–22 Spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) as described previously [17]. Anisotropy measurements were carried out using Glen-Thompson polarization accessory in the same instrument and values were calculated as described previously [18].

Time-Resolved Fluorescence Measurements

Time-resolved fluorescence intensity decays were utilized to calculate fluorescence lifetimes using an IBH 5000F NanoLED equipment (Horiba Jobin Yvon, NJ) with Data Station software in the time-correlated single photon counting (TCSPC) mode as described previously [18] with some modifications. The excitation source used was a pulsed light emitting diode (NanoLED-17) which generates an optical pulse at 295 nm and pulse duration less than 1.2 ns, and is run at 1 MHz repetition rate. A nominal bandpass of 4 nm in emission slits were used for all experiments. Intensity-averaged mean lifetimes were calculated using the following equation [19]:

$$\langle \tau \rangle = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2 + \alpha_3 \tau_3^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2 + \alpha_3 \tau_3} \quad (1)$$

from pre-exponential factor and lifetime components (α_i and τ_i) obtained upon fitting of the fluorescence intensity decay to a triexponential function.

Circular Dichroism Measurements

CD measurements were performed at room temperature (~23 °C) on a Chirascan Plus Spectropolarimeter (Applied Photophysics, Surrey, UK) calibrated with (+)-10-camphorsulfonic acid [20]. Spectra were scanned using a

quartz optical cell having a path length of 0.1 cm and recorded in 0.2 nm wavelength increments with a band width of 3 nm and an integration time of 2 s. To explore any changes in the secondary structure of pHLIP, spectra were scanned in the far-UV range from 190 to 260 nm. Subtraction of proper blanks from the measured spectra ensured that the background was corrected. Data are represented as mean residue ellipticities and were calculated using the equation:

$$[\theta] = \theta_{\text{obs}} / (10Cl) \quad (2)$$

where l is the path length in cm, C is the concentration of peptide bonds in mol/l and θ_{obs} is the observed ellipticity in mdeg. The CD spectra of pHLIP in solution were deconvoluted with the web-based CD analysis resource, DichroWeb [21, 22], using the CONTIN analysis method [23] with SMP180 [24] as the reference data set. In addition, for purpose of representation, a modest degree of smoothing of the CD spectra was done in Microcal Origin version 8.0 (OriginLab, Northampton, MA) using the adjacent averaging program, while making sure that the overall shape of spectra remained unchanged.

Statistical Analysis

Student's two-tailed unpaired t -test was performed to estimate significance levels using Graphpad Prism software, version 4.0 (San Diego, CA). Plots were generated using Microcal Origin software, version 6.0 (OriginLab, Northampton, MA).

Results and Discussion

pHLIP is a hydrophobic peptide which shows significant conformational diversity depending on pH and local environment. Due to the hydrophobic nature of pHLIP, the peptide is prone to aggregation and previous work has shown that pHLIP aggregates beyond a concentration of ~7 μ M [2]. Although pHLIP has been used for translocation of cargo across the cell membrane [6–8], a basic understanding of the aggregation behavior of pHLIP at low peptide concentrations, necessary for biophysical analysis of membrane interaction, is still lacking. In this work, we have utilized fluorescence-based approaches and CD spectroscopy to characterize the monomeric and aggregated forms of pHLIP in solution at low concentrations. The concentrations corresponding to monomeric and aggregated pHLIP were 3 and 12 μ M, respectively, in all experiments.

The amino acid sequence of pHLIP features two tryptophan residues at positions 9 and 15 (Fig. 1a). The intrinsic fluorescence of these tryptophans can be exploited to monitor the microenvironment around these residues in a label-free manner. To explore any difference in the microenvironment between the monomeric and aggregated forms of pHLIP, we

used the red edge excitation shift (REES) approach. REES is defined as the shift in the wavelength of emission maximum of fluorescence toward higher wavelengths, induced by shifting the excitation wavelength toward the red edge of the absorption spectrum [25–27]. REES is observed with polar fluorophores (with non-zero ground state dipole moment) in dynamically constrained environment, as experienced in viscous media such as the membrane interface. In such viscous environment, the rate of solvent relaxation (reorientation) is slow relative to fluorescence lifetime, resulting in REES [25]. REES therefore is a unique approach as it offers information on slow solvent (dipole) dynamics in the excited state. Since the dynamics of hydration is related to protein function, REES has emerged as an important tool to monitor the organization and dynamics of soluble and membrane proteins [26].

Fig. 1a shows the representative intensity-normalized emission spectra of monomeric and aggregated pHLIP. The excitation wavelength used was 295 nm in order to selectively excite only the tryptophan residues [28]. The emission maximum of monomeric and aggregated pHLIP was found to be ~347 and 346 nm, respectively. Fig. 1b shows REES data for monomeric and aggregated pHLIP. The emission maximum of monomeric pHLIP exhibited a shift from 347 to 351 nm, upon increasing the excitation wavelength from 280 to 307 nm. This corresponds to REES of 4 nm (shown in the inset in Fig. 1b). In case of aggregated pHLIP, the emission maximum displayed a shift from 346 to 352 nm, giving rise to an increased REES of 6 nm (inset of Fig. 1b). The increase in magnitude of REES in case of the aggregated peptide is indicative of motional restriction caused by aggregation. However, the increased magnitude of REES in the aggregated form would represent a lower limit since it would be a weighted average of aggregated pHLIP coexisting in equilibrium with monomeric pHLIP. It is important to note that a significant difference ($p < 0.05$) was found in the magnitude of REES between aggregated and monomeric pHLIP. As a control, we carried out REES measurements of pHLIP denatured in 8 M urea. Interestingly, denatured pHLIP exhibited no REES with an emission maximum of 349 nm, independent of the excitation wavelength (see Fig. 1b). The difference in the magnitude of REES between denatured and monomeric pHLIP was also found to be statistically significant ($p < 0.001$).

It is known that fluorescence anisotropy depends on the excitation wavelength in motionally restricted media [29]. A subpopulation of fluorophores gets selectively excited upon excitation at the red edge. This subpopulation of fluorophores interacts strongly with polar solvent molecules in the excited state, thereby leading to slower rotational mobility and increased anisotropy of these ‘solvent-relaxed’ fluorophores. The change in fluorescence anisotropy with increasing excitation wavelength for monomeric and aggregated pHLIP are shown in Fig. 2a. As shown in the figure, the anisotropy increases upon increasing the excitation wavelength from 280 to 307 nm in both cases. This type of excitation wavelength

dependent change in fluorescence anisotropy is characteristic in case of fluorophores in motionally restricted environment [29]. Fig. 2b shows the change in fluorescence anisotropy for monomeric and aggregated pHLIP with increasing emission wavelength. In both cases, we observed a progressive decrease in anisotropy across the emission spectrum, typically observed for fluorophores in restricted media [29]. Interestingly, the anisotropy of aggregated pHLIP was lower than the corresponding value of anisotropy for monomeric peptide across the emission spectrum. This could be due to self (homo) energy transfer between tryptophans in pHLIP monomers in the aggregate.

In order to explore the environment around pHLIP tryptophan residues, we measured fluorescence lifetimes under

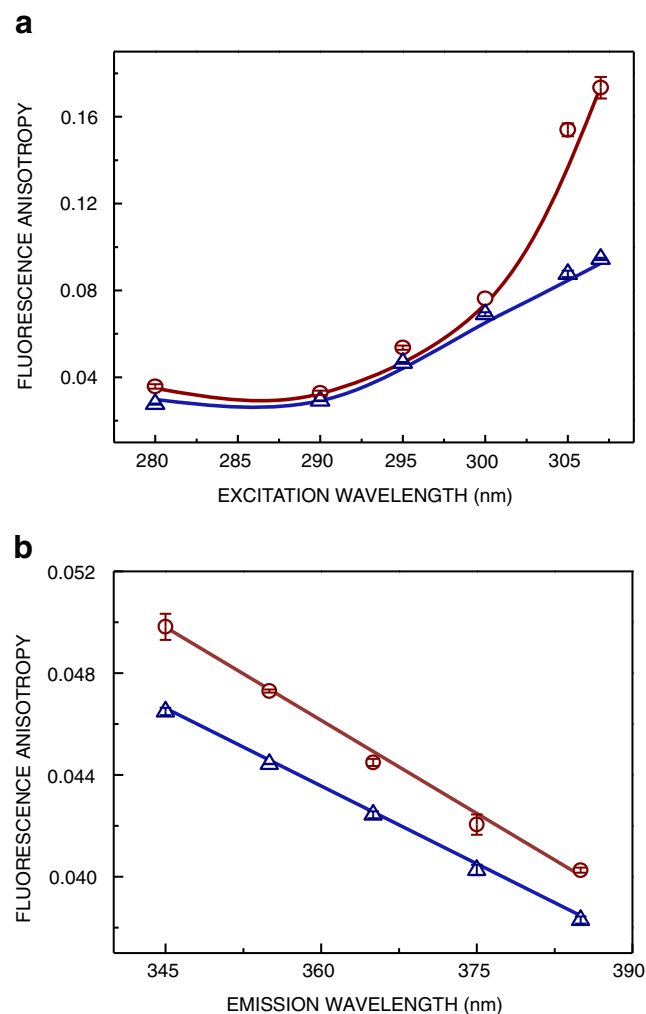


Fig. 2 (a) Fluorescence anisotropy of tryptophan with increasing excitation wavelength in monomeric (○) and aggregated (Δ) forms of pHLIP. The emission wavelength used was 346 nm. (b) Fluorescence anisotropy of tryptophan with increasing emission wavelength in monomeric (○) and aggregated (Δ) forms of pHLIP. The excitation wavelength used was 295 nm. All other conditions are as in Fig. 1. Data points shown are means $\pm$ SE of at least three independent measurements. Lines joining the data points serve only as viewing guides. See Experimental for further details

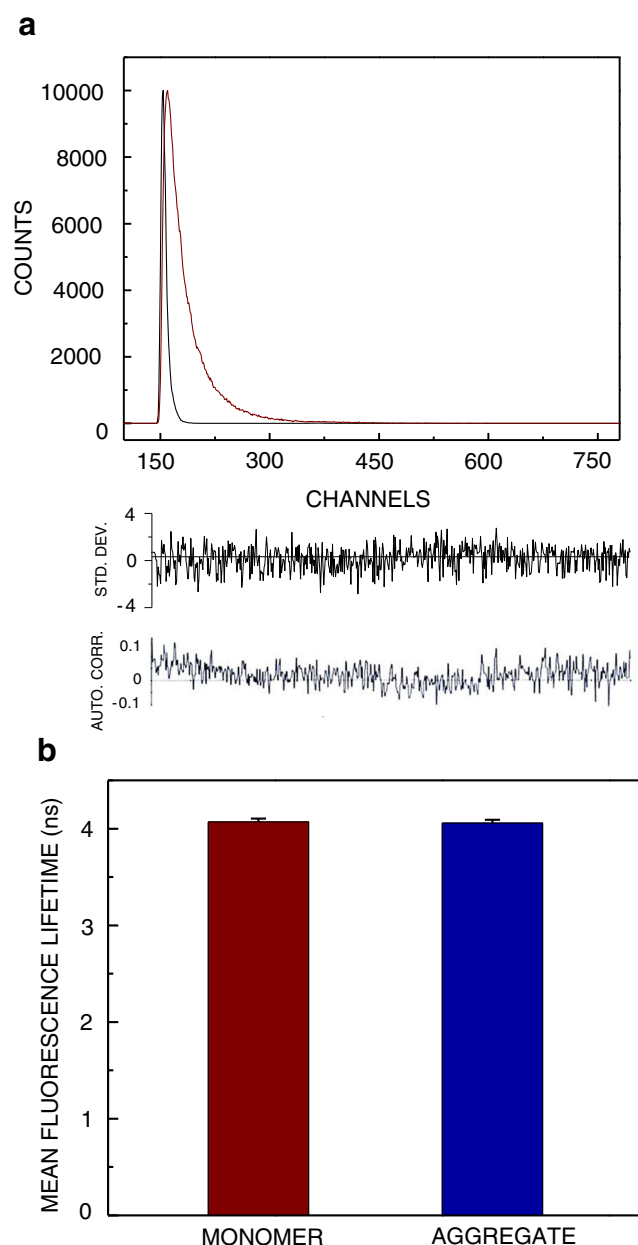


Fig. 3 (a) Representative time-resolved fluorescence intensity decay of monomeric pHLIP at pH 8. The excitation wavelength of the pulsed diode light source was 295 nm and emission was monitored at 346 nm. The profile of the pulsed light emitting diode (LED) is represented by the narrow peak on the left. The decay profile (fitted to a triexponential function) corresponds to the comparatively wide peak toward the right. The weighted residuals and the autocorrelation function of the weighted residuals are shown in the two plots below. (b) Mean fluorescence lifetimes of tryptophan in monomeric (left bar) and aggregated (right bar) forms of pHLIP. Mean fluorescence lifetimes were calculated using Eq. (1). All other conditions are as in Fig. 1. Data points shown are means $\pm$ SE of at least three independent measurements. See Experimental and Table 1 for further details

these conditions. A typical decay profile of pHLIP tryptophan fluorescence with its triexponential fitting is shown in Fig. 3a. Fluorescence decays obtained could be best fitted to a triexponential function. Intensity-averaged mean fluorescence

Table 1 Representative fluorescence lifetimes of tryptophan for monomeric and aggregated pHLIP

Condition	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)
Monomeric pHLIP	0.13	0.61	0.56	2.55	0.31	5.55
Aggregated pHLIP	0.13	0.66	0.50	2.46	0.37	5.22

The excitation wavelength was 295 nm and emission was monitored at 346 nm in all cases. All other conditions are as in Fig. 1. See Experimental for further details

lifetime was used as it does not depend on the analysis method and the number of exponentials used to fit the time-resolved fluorescence decay. The fitted lifetime components of tryptophan in monomeric and aggregated pHLIP are shown in Table 1. The mean fluorescence lifetimes of pHLIP tryptophans were calculated from data in Table 1 using eq. (1) and are shown in Fig. 3b. The figure shows that the mean fluorescence lifetime of pHLIP was ~ 4 ns, for both monomeric and aggregated forms. This ensures that the anisotropy values shown in Fig. 2 are not affected by fluorescence lifetime.

Figure 4 shows the CD spectra of pHLIP in monomeric and aggregated forms. As evident from the figure, the peptide predominantly adopts a random coil conformation at both concentrations, although a minor shift toward longer wavelengths was observed in the CD spectrum in the aggregated form. These results are in overall agreement with previous measurements [2], although the latter were carried out at much higher (mM) peptide concentrations. Interestingly, there was $\sim 8\%$ increase in α -helical content in aggregated pHLIP relative to its monomeric form (data not shown). Our results imply that aggregation promotes secondary structure formation in pHLIP, also apparent from the higher magnitude of REES in the aggregated peptide (Fig. 1b).

Aggregation of membrane-active peptides is an interesting aspect in terms of their function, since depending on the situation,

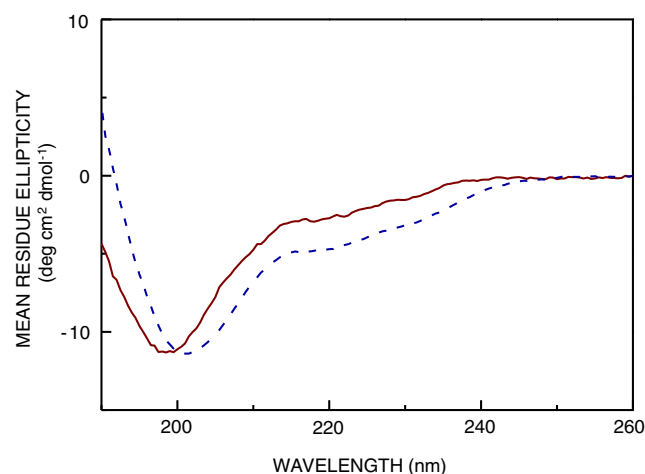


Fig. 4 Far-UV CD spectra of monomeric (—) and aggregated (---) forms of pHLIP in 10 mM phosphate buffer at pH 8. All other conditions are as in Fig. 1. See Experimental for further details

aggregation could compete with membrane interaction and could even enhance this interaction. However, monitoring aggregation of such hydrophobic peptides is often challenging. In this work, we have utilized fluorescence-based approaches and CD spectroscopy to characterize the monomeric and aggregated forms of pHLIP in aqueous solution at low concentrations ($\sim\mu\text{M}$ range). The aggregation behavior of pHLIP in aqueous solution was previously studied by Reshetnyak et al. [2]. Although these authors detected aggregation at low ($\sim\mu\text{M}$) concentrations, all the fluorescence measurements were carried out at very high ($\sim\text{mM}$) concentrations of the peptide. In contrast, we chose to monitor fluorescence signature of the peptide in $\sim\mu\text{M}$ concentration range since peptide aggregation under these conditions could be experimentally relevant. Our results show that pHLIP aggregates even at such low concentrations, as evident from differential REES and CD signatures. The knowledge and understanding of the aggregation behavior of pHLIP will be useful for monitoring its interactions with membranes.

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Author Contributions B.D.R. performed the experiments, H.C. helped in data analysis, S.K. provided the peptide, B.D.R. and A.C. wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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