The Effect of Interactions Involving Ionizable Residues Flanking Membrane-Inserted Hydrophobic Helices upon Helix−Helix Interaction†

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ABSTRACT: We examined the effect of ionizable residues at positions flanking the hydrophobic core of helix-forming polyLeu peptides upon helix−helix interactions within model membrane vesicles composed of dioleoylphosphatidylcholine. The peptides studied were flanked on both the N and C termini either by two Lys (K_2-flanked peptide), one Lys plus one Asp (DK-flanked peptide), or one Lys plus three Asp (KD_3-flanked peptide). The fluorescence of a Trp residue positioned at the center of the hydrophobic sequence was used to evaluate peptide behavior. As judged by the concentration dependence of the maximum wavelength of Trp emission, there was significant oligomerization of the KD_3- and DK-flanked peptides, but not the K_2-flanked peptide, at neutral pH. At neutral pH mixtures of K_2- and KD_3-flanked peptides associated with each other, but mixtures of the K_2- and DK-flanked peptides did not. Oligomerization by the DK- and KD_3-flanked peptides decreased under low pH conditions in which the Asp residues were protonated. Additional experiments showed that at neutral pH the KD_3-flanked peptide showed an increased tendency to oligomerize when as little as 10−15 mol % of an anionic lipid, phosphatidylglycerol, was present. The behavior of the other peptides was not strongly influenced by phosphatidylglycerol. These results can largely be explained by modulation of helix−helix interactions via electrostatic interactions involving the helix-flanking ionizable residues. Such interactions may influence membrane protein folding. The self-association of anionic KD_3-flanked peptides suggests that additional interactions involving charged residues also can modulate helix−helix association.

Integral membrane proteins perform a variety of important biological functions, and it is estimated that 30% of a genome can encode these proteins (*1*). The majority of integral membrane proteins are composed of transmembrane (TM)[1] α-helical bundles. Therefore, it is important to understand how the amino acid sequence of TM α-helices controls their folding and structure. Some important information about membrane protein amino acid composition is already understood. Hydrophobic residues predominate in those parts of a TM helix that are buried in the membrane and exposed to lipid hydrocarbon chains, whereas polar residues are less abundant in these segments (*2*).

Ionizable ("charged") residues are relatively rare in the core of TM helices (*2*). Clearly, an energetic cost must be paid for burying ionizable residues in the nonpolar environment of a bilayer, but this is not necessarily due to burial of a charged group. When close to the bilayer, surface ionizable residues may carry a charge (*3*), but it appears that deeply buried ionizable residues remain in their uncharged ionization state, with their deep location destabilized by their polarity and the energetic cost of maintaining an uncharged state (*4*). In any case, relative to location in the bilayer core, it is energetically less costly to place ionizable residues at the polar/interfacial region of a bilayer and least costly to place them in the aqueous solution beyond the bilayer (*2*, *3*, *5*, *6*).

Relatively little is known about how ionizable residues at the edge of TM helices affect helix behavior. It has been suggested that flanking ionizable residues act as anchors, i.e., fix the position of the edge of the hydrophobic segment of a helix relative to the bilayer (*7*). An anchoring residue can be defined as one that has a favorable interaction with the interfacial region of the bilayer relative to its interaction with aqueous solution or the core of the bilayer (e.g., Trp). However, even a residue that prefers to locate in solution relative to the bilayer interface will still be able to assist in anchoring if it has a lower energy at the bilayer surface than it has when in the hydrophobic core of the bilayer. Asp and Lys seem to fall into this class of residues (*5*, *8*), and at least Lys seems to have significant anchoring abilities (*8*). However, strong anchoring may not be characteristic of all charged residues, because Arg does not show strong anchoring effects (*8*).

A different function of positively charged amino acids located in or near regions flanking hydrophobic helices is their proposed action as topological determinants of the TM helix orientation. Their behavior gives rise to the "positive inside" rule, which states that positively charged residues

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[1] Abbreviations: DK-flanked peptide, acetyl-DKGL_9WL_9KDA-amide; DOPC, dioleoyl-sn-glycero-3-phosphocholine, dioleoylphosphatidylcholine; DOPG, dioleoyl-sn-glycero-3-phosphoglycerol, dioleoylphosphatidylglycerol; 10-DN, 10-doxylnonadecane; K_2-flanked peptide, acetyl-K_2GL_9W$L_9$$K_2$A-amide; KD_3-flanked peptide, acetyl-KD_3L_9W$L_9$$D_3$K-amide; TM, transmembrane; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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are frequently found on the cytoplasmic side of the membrane (*9*).

One other function of helix-flanking ionizable residues may be in controlling TM helix−helix interaction (*10*, *11*). This has been little studied in contrast to the control of helix−helix interaction by van der Waals and polar interactions between residues in the core of TM helices (*12*−*15*). We have approached this question with polyLeu peptides, which are extremely hydrophobic peptides that come from TM helices (*4*, *16*−*18*). We found previously that the ionization state of Lys residues flanking a polyleucine helix modulate the ability of such helices to oligomerize within bilayers (*4*). In the present study, combinations of Asp and Lys residues are placed in the positions flanking polyLeu peptides. The results show that the nature of the flanking residues, electrostatic interactions between peptides, and electrostatic interactions between peptide and lipid can significantly influence the degree to which TM helices within membranes interact with each other.

EXPERIMENTAL PROCEDURES

Materials. The polyLeu peptides $K_2GL_9WL_9K_2A$, $KD_3L_9WL_9D_3K$, $K_2GL_9YL_9K_2A$, and $DKGL_9WL_9KDA$ were purchased from Research Genetics division of Invitrogen (Huntsville, AL). All the peptides had acetylated N-termini and amide blocked C-termini. Peptides were purified by reverse-phase HPLC using a C18 column as described previously (*16*, *19*). A binary mobile phase composed of mixtures of 0.5% trifluoroacetic acid/2-propanol and 0.5% trifluoroacetic acid/H_2O (v/v) was used for purification. Elution was achieved by using an increasing gradient of 2-propanol. MALDI-TOF mass spectrometry was used to confirm the purity of the peptides. Final purity was roughly estimated to be 85−95%. Peptide concentration was determined from the UV absorbance at 280 nm by using $\epsilon = 5500\ M^{-1}\ cm^{-1}$. Peptides were stored in ethanol at 4 °C. Dioleoyl-sn-glycero-3-phosphocholine (dioleoylphosphatidylcholine, DOPC) and dioleoyl-sn-glycero-3-phosphoglycerol (dioleoylphosphatidylglycerol, DOPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids were stored in chloroform at −20 °C. As judged by thin-layer chromatography on silica gel plates run in 65:25:4 (v/v) chloroform:methanol:water, some preparations of DOPG contained a few percent of a breakdown product. Acrylamide was purchased from Sigma Chemical (St. Louis, MO). 10-Doxylnonadecane (10-DN) was purchased from Aldrich Chemical (Milwaukee, WI). (Discontinued; contact authors for availability.) Its concentration was determined by dry-weight analysis. It was stored as a stock solution in ethanol at −20 °C. All other chemicals were reagent-grade.

Fluorescence Measurements. Trp fluorescence was measured on a SPEX τ2 fluorolog spectrofluorometer operating in steady-state mode. Generally, fluorescence measurements were made in a semimicro quartz cuvette having a 1 cm excitation path length and 4 mm emission path length. For samples in which pH was titrated, measurements were made in a cuvette with a 1 cm path length for both excitation and emission. For most experiments, a 2.5 mm (nominal bandwidth 4.5 nm) excitation slit and a 5.0 mm emission slit (bandwidth 9 nm) were used. For the pH titration experiments, a 5.0 mm excitation slit and a 5.0 mm emission slit were used. The excitation wavelength was 280 nm unless stated otherwise. Emission spectra were recorded from 300 to 360 or 380 nm. Estimated λ_{max} values obtained from duplicate samples were generally reproducible to ±1 nm.

pH Titration Experiments. Trp fluorescence was studied as a function of pH in bilayer vesicles made by dilution from ethanol. This procedure results in formation of unilamellar vesicles (*4*). The appropriate aliquots of peptides and lipids dissolved in organic solvent were mixed, dried under nitrogen, and then redissolved in 40 μL of ethanol. Next, the samples were diluted with 1960 μL of 10 mM sodium phosphate,150 mM NaCl, pH 7.6. The final peptide concentration was 2 μM, and the final lipid concentration was 200 μM. Duplicate samples were prepared. Background samples lacking peptide were also prepared. The pH in each sample was lowered to the desired starting value for the titration (about pH 4) by adding a 4−5 μL aliquot of glacial acetic acid. Emission spectra were then recorded, generally from 300 to 360 nm. To increase pH, successive aliquots of 0.5−2.0 M NaOH were added. After each addition the samples were incubated for approximately 90 s, and the emission spectra then remeasured. It should be noted that the reported data is that after intensity of background samples was subtracted, and corrected for dilution of peptide-containing samples during the titration.

Acrylamide Quenching Measurements. To quantify acrylamide quenching, fluorescence of samples containing model membrane-incorporated peptides, or background samples lacking peptide, was measured both before and after the addition of a 50 μL aliquot of acrylamide from a 4M stock solution dissolved in water. Samples were prepared as described above, except that all volumes were decreased 2-fold. Fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. Corrections were made for both dilution by the addition of acrylamide and for inner filter effect. Inner filter corrections were done by multiplying measured fluorescence intensity by the formula $10^{(\epsilon 0.5bC)}$ where ϵ is the molar extinction coefficient of acrylamide at 295 nm in units $M^{-1}\ cm^{-1}$, b is the cuvette excitation path length in cm, and C is the acrylamide concentration in the sample in M.

10-Doxylnonadecane Quenching Measurements. To measure the efficiency of 10-DN quenching, the fluorescence of samples in the absence of 10-DN was compared to that in its presence. Samples containing model membrane-incorporated peptides or background samples without peptide were prepared as noted above except that some samples contained 10 mol % 10-DN under conditions in which the lipid + 10DN concentration was 200 μM. Fluorescence was measured with an excitation wavelength of 280 nm and emission wavelength of 330 nm.

Calculation of the Acrylamide to 10-DN Quenching Ratio (Q-Ratio). The ratio of quenching by acrylamide to that by 10-DN (Q ratio) was used to estimate Trp depth in the membrane. Q-ratio was calculated from the formula: $Q\ ratio = [(F_o/F_{acrylamide}) - 1]/[(F_o/F_{10-DN}) - 1]$, where F_o is sample fluorescence with no quencher and $F_{acrylamide}$ and F_{10-DN} are the fluorescence intensities in the presence of acrylamide and 10-DN, respectively (*23*).

Effect of Varying Peptide to Lipid Ratio by Increasing Lipid Concentration on Emission λ_{max}. To examine whether peptide oligomers are formed within vesicles, Trp λ_{max} was

measured as a function of the lipid:peptide ratio. A peptide λ_{max} dependent on lipid:peptide ratio is indicative of oligomerization (*17*). Samples containing peptide and DOPC were made by ethanol dilution similar to that described for the pH titration experiments. For these experiments, the peptide concentration (2 μM) was kept constant while the lipid concentration was varied between 100 and 1500 μM. Samples were made by dissolving dried mixtures of peptides and lipids in 20 μL of ethanol and then diluting to 1 mL with 10 mM sodium phosphate, 150 mM NaCl, pH 7.6. Duplicate samples were prepared. In addition, background samples lacking peptide were prepared.

Bromination of Tyrosine-Containing Peptide. A 0.1−0.3 μmol sample of $K_2GL_9YL_9K_2A$ peptide (Tyr-containing K_2-flanked peptide) dissolved in 500 μL methanol was dibrominated on tyrosine by adding a total of 5.25 mol of Br_2 per mol of peptide. The bromine, from a 1:1000 v/v Br_2:methanol solution, was mixed with the peptide in three equal aliquots added 10 min apart. The sample mixture was then dried under N_2, dissolved in 4:6 v/v 2-propanol:water, and purified by HPLC, as described above. This separated unbrominated and monobrominated peptides from the dibrominated peptide. Purity was confirmed by MALDI-TOF. Concentration of dibrominated peptide was determined spectroscopically, using an $\epsilon_{284\ nm}$ of 2616 cm^{-1} M^{-1} obtained experimentally from the absorbance of pure dibromotyrosine (Sigma Chemical).

Dibromotyrosine Quenching Experiments. Samples of peptides incorporated into lipid vesicles were prepared by ethanol dilution, similar to the procedure described above, to a final volume of 800μL using 10mM Na phosphate, 150 mM NaCl, pH 6.9. Samples for quenching experiments with dibromoTyr-labeled K_2-flanked peptide contained 200 μM lipid and 2 μM of either K_2 -or KD_3 -flanked peptide combined with 1μM of the dibromoTyr-labeled K_2-flanked peptide (F samples) or without dibromoTyr-labeled peptide (Fo samples). When desired, the pH of these samples was adjusted with glacial acetic acid or a NaOH solution. Fluorescence emission spectra were recorded over the range 300−375 nm, with excitation at 280 nm. Background spectra from samples lacking peptides were subtracted from Fo samples, and backgrounds containing both lipid and dibromoTyr-labeled peptide were subtracted from the F samples. Spectra were also corrected for dilution by either acetic acid or NaOH. The ratio of intensities in the samples with quencher to that without quencher (F/F_o) was calculated from the intensity values at 330 nm.

Circular Dichroism Measurement of Peptide Secondary Structure. Secondary structure of the peptides in bilayer vesicles was studied with circular dichroism (CD). The ethanol dilution method described above was employed to prepare l mL samples containing 2 μM peptide and 200 μM DOPC. A buffer composed of 1 mM sodium phosphate, 15 mM NaCl, pH 7.6, was used. This contained only 10% of the NaCl concentration used for fluorescent measurements because higher salt concentrations interfere with CD measurements. Other studies show the use of diluted buffer does not strongly influence secondary structure (G. A. Caputo and E. London, unpublished observations). CD was measured at room temperature using a JASCO J-715 CD spectrometer and 1 mm path length quartz cuvettes. Generally, the final spectra were the average of at least 40 scans taken at 50 nm/min. The program SELCON3 was employed to estimate the α-helical content of the peptides (*20*). Background spectra of buffer and lipid alone (i.e., without peptide) were subtracted before analysis of secondary structure.

RESULTS

The pH-Dependent Fluorescence of Various polyLeu Peptides in DOPC Vesicles. We wished to understand the effect of the charge on residues flanking the hydrophobic core of TM helices upon the behavior of helices in membranes. To do this we studied three different hydrophobic polyLeu peptides incorporated into model membrane vesicles. One peptide was flanked by 2 Lys residues at each end (K_2-flanked peptide), a second by 1 Lys and 1 Asp residue (DK-flanked peptide), and the third by 1 Lys and 3 Asp residues (KD_3-flanked peptide). All three peptides had a Trp at the center of their hydrophobic sequence and blocked termini. Our first aim was to identify the pH values at which different ionized states would exist. To do this the pH dependence of Trp fluorescence parameters likely to be sensitive to the ionization state of vesicle-incorporated peptides was studied. The pH dependence of Trp λ_{max}, the ratio of fluorescence emission at 350 nm to that at 330 nm (F350/330) and emission intensity at 330 nm were measured. When a Trp is in a polar environment, it gives red-shifted fluorescence in which it shows a high λ_{max} and high F350/330, whereas in a nonpolar environment, it gives blue-shifted fluorescence with a low λ_{max} and low F350/330. (Despite the similarity of these latter parameters, both λ_{max} and F350/330 were monitored because λ_{max} is widely reported, but F350/330 can be slightly more sensitive to small shifts in emission, and is less ambiguous when emission maxima are broad (*21*).) Intensity at a single wavelength was measured because it also can be sensitive to local environment.

Figure 1 shows the pH dependence of the fluorescence properties of these peptides when then are incorporated into lipid vesicles composed of dioleoylphosphatidylcholine (DOPC). The K_2-flanked peptide showed a pH dependence of fluorescence behavior very similar to that we reported for this peptide in a previous study (*4*). As shown in Figure 1, from pH 4 to 9 this peptide exhibited a pH-independent, blue-shifted λ_{max}, low F350/330 ratio, and pH-independent intensity. Above pH 9, there was a gradual red shift in Trp fluorescence and decrease in fluorescence intensity. As described in our previous study, this red-shiftresults from deprotonation of the flanking Lys residues (*4*).

The fluorescence of the KD_3-flanked peptide shows a different pH dependence. As shown in Figure 1, at low pH (pH 4) the KD_3-flanked peptide shows a relatively blue-shifted λ_{max}, low F350/330 ratio, and relatively high fluorescence intensity similar to the K_2-flanked peptide. However, as the pH is raised from 4 to 5.5, Trp λ_{max} red-shifts, F350/330 increases, and fluorescence intensity decreases. The λ_{max} remains constant over the pH range of 5.5−10.5. However, F350/330 shows a hint of a maximum, and fluorescence intensity at 330 nm showing a distinct minimum, near pH 6. Above pH 10−10.5, Trp λ_{max} blue shifts, and F350/330 decreases.

These pH-dependent changes can also be assigned to changes in amino acid ionization. At the lowest pH values, all of the flanking residues should be protonated, so that Lys

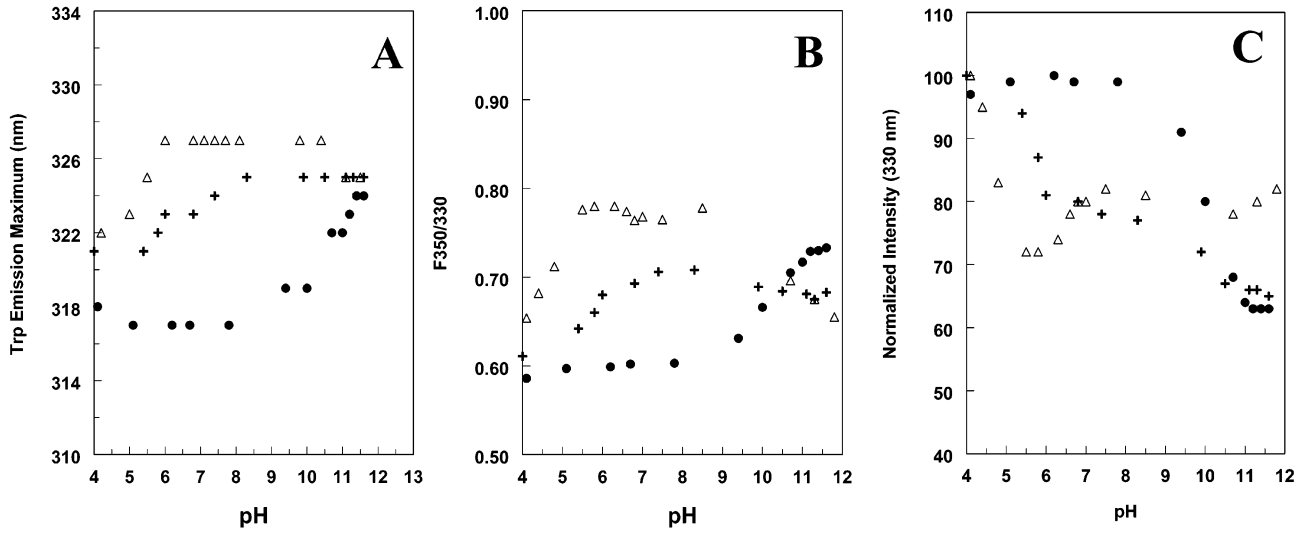

FIGURE 1: Trp emission properties of the K_2-flanked, DK-flanked, and the KD_3-flanked peptides in DOPC vesicles vs pH. (A) Trp fluorescence emission λ_{max} B. Ratio of Trp emission intensity at 350 nm to that at 330 nm (F350/330). (C) Trp emission intensity at 330 nm normalized to 100% at the point of maximum intensity. (•) K_2-flanked, (+) DK-flanked, and (Δ) KD_3-flanked peptides. Samples contained 2 μM peptide and 200 μM lipid. The results of single titrations are shown. In each case duplicate titrations giving similar results were obtained.

residues are charged while Asp residues are uncharged. The changes between pH 4 and 7 are likely to be associated with the ionization of the Asp groups because the pK_a for Asp near the bilayer surface should fall in this range (see Discussion). However, the Asp residues at different positions are likely to slightly different pK_a values because each should reside at different depths relative to the bilayer surface and because the charge on Asp residues that deprotonate first should electrostatically inhibit the deprotonation of neighboring Asp residues. Thus, the apparent maximum in local polarity around the Trp at pH 6 may correspond to a state in which there has been deprotonation of some, but not all Asp residues, so that the peptide has close to neutral net charge (see Discussion). Between pH 7 and 10, it is likely that all the Asp residues have deprotonated and the changes above pH 10 are undoubtedly associated with deprotonation of the flanking Lys residues.

The pH dependence of Trp fluorescence was also measured for the DK-flanked peptide. Like the KD_3-flanked and K_2-flanked peptides, the DK-flanked peptide shows a blue-shifted λ_{max}, low F350/330 ratio, and high fluorescence intensity at 330 nm at pH 4 (Figure 1). As pH is increased from 5 to 7, λ_{max} red-shifts, F350/330 increases, and intensity at 330 nm decreases. This is also similar to the behavior of the KD_3-flanked peptide, although the red shift in λ_{max} and corresponding increase in F350/330 is less for the DK-flanked peptide. At higher pH, there is little change in λ_{max} and only a small decrease in F350/330. However, the intensity at 330 nm shows a distinct sigmoidal decrease as the pH is increased from 8 to 10.5. These changes can also be assigned to the pH-dependent ionization events. Deprotonation of the Asp residues is associated with the change in fluorescence properties between pH 5 and 7, while the changes between pH 9 and 10.5 are associated with the deprotonation of the Lys residues.

The pH Dependent Behavior of the KD_3-Flanked Peptide in Vesicles Containing 20% DOPG. Because peptide behavior was strongly affected by amino acid charge, it was of interest to examine how negatively charged phospholipids, which are present in natural membranes, affected peptide behavior. To do this, dioleoylphosphatidylglycerol (DOPG), which has a negative charge throughout the pH range studied, was incorporated into the DOPC-containing vesicles at a concentration of 20mol %. This fraction of anionic lipid is comparable to that found in cell membranes (*22*). Again our first study was simply aimed at identifying pH values at which different ionization states occur.

Figure 2 shows the pH dependence of fluorescence properties for the K_2-flanked, KD_3-flanked, and DK-flanked peptides when incorporated into vesicles composed of 20mol % DOPG/80mol % DOPC. The behavior for all of the peptides is, in general, very similar to that in vesicles composed of DOPC. However, there are some differences. One is a shift in apparent pK_a values. There is a roughly 1 pH unit upward shift in the pH at which the Asp and Lys residues deprotonate for each peptide. This shift in pK_a can be explained by the fact that DOPG imparts a negative charge to the vesicles and attracts a high proton concentration near the surface of anionic vesicles. These factors tend to stabilize the positive charge on Lys residues and destabilize negative charge on Asp residues.

A second difference is that near neutral pH values the KD_3-flanked peptide exhibits more red-shifted fluorescence as judged by λ_{max} and higher F350/330 in 20% DOPG vesicles than those in 100% DOPC vesicles (compare Figures 1 and 2). Lipid composition has smaller effects on the Trp fluorescence emission maxima for the DK-flanked and K_2-flanked peptides.

It should be noted that the effects of 20mol % DOPA, another anionic lipid, on the behavior of the KD_3-flanked peptide were similar to those with DOPG (data not shown). This is consistent with an electrostatic origin of the effects arising from the presence of DOPG.

The Effect of Fraction of DOPG in Bilayers on Trp λ_{max} of the KD_3-Flanked Peptide. The results described above show that the presence of DOPG in vesicles influences the behavior of the KD_3- flanked peptide. To see whether this behavior could be observed at lower levels of an anionic lipid, this peptide was incorporated into vesicles containing varying amounts of DOPG at neutral pH. The results are

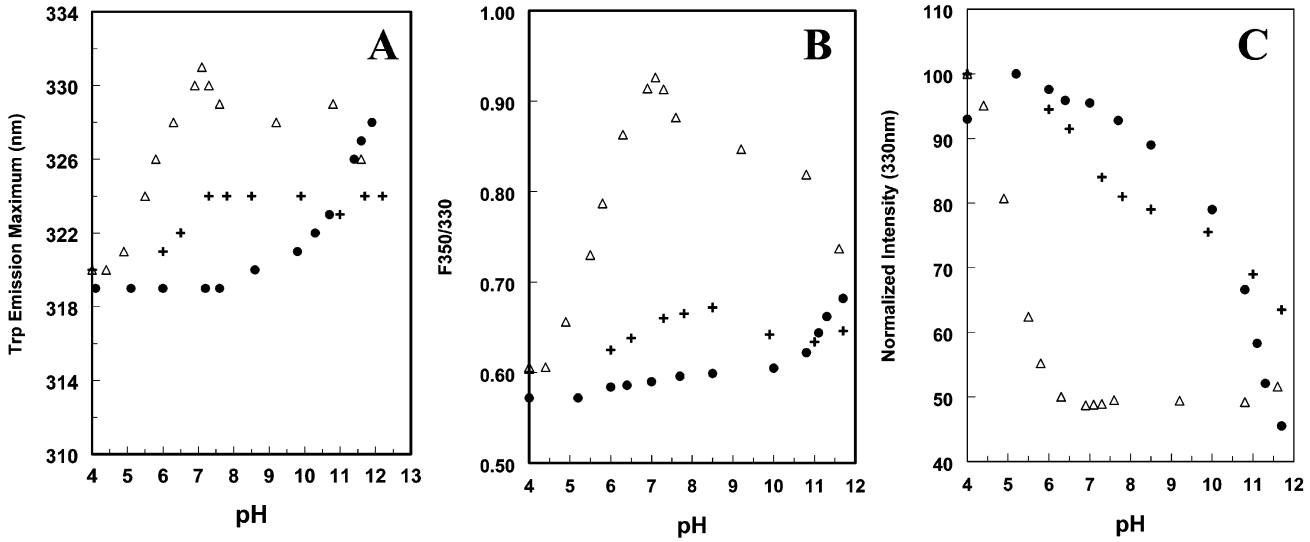

FIGURE 2: Trp emission properties of the K_2-flanked, DK-flanked, and KD_3-flanked peptides in 20 mol %DOPG/80 mol %DOPC vesicles vs pH. (A) Trp fluorescence emission λ_{max}. (B) Ratio of Trp emission intensity at 350 nm to that at 330 nm (F350/330). (C) Trp emission intensity at 330 nm normalized to 100% at the point of maximum intensity. (•) K_2-flanked, (+) DK-flanked, and (△) KD_3-flanked peptides. Samples contained 2 μM peptide and 200 μM lipid. The results of single titrations are shown, but duplicate titrations gave similar results.

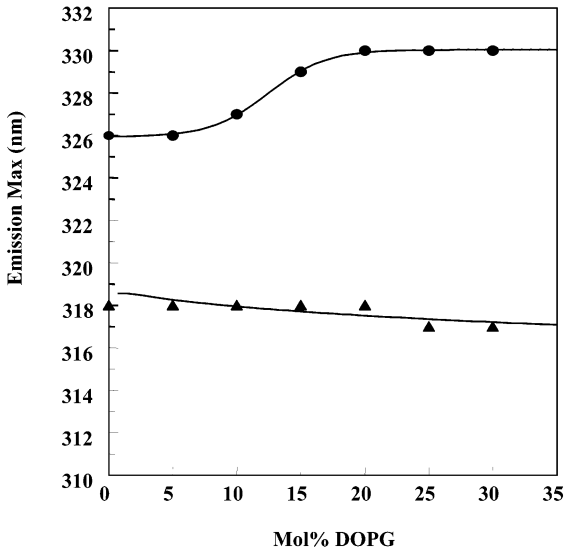

FIGURE 3: The effect of varying the amount of DOPG within vesicles on Trp emission maximum of K_2-flanked and KD_3-flanked peptides. Samples contained 2 μM of (▲) K_2-flanked or (●) KD_3-flanked peptides incorporated into various 200 μM mixtures of DOPG and DOPC dispersed in 10 mM sodium phosphate, 150 mM NaCl, pH 7.6. Average values from duplicate experiments are shown. λ_{max} values were reproducible to within ±1 nm.

presented in Figure 3, which shows that the Trp λ_{max} exhibits a sigmoidal increase as the amount of DOPG in the vesicle is increased from 0 to 20mol % DOPG and stabilizes above 20 mol %. In contrast, DOPG does not have a significant effect on Trp λ_{max} of the K_2-flanked peptide at neutral pH over the entire concentration range studied.

Interpretation of the pH Dependence of λ_{max} in Terms of Peptide Behavior. Location of the K_2-Flanked, DK-Flanked, and KD_3-Flanked Peptides in the Lipid Bilayer. The experiments above identified the pH at which different ionization states exist for each peptide and revealed the existence of Trp fluorescence-detected structural/conformational differences dependent upon sequence, pH and anionic lipid. Our further studies were aimed at revealing the nature of these differences.

The λ_{max} values were an important clue on this regard. All three peptides have a Trp at the center of the hydrophobic sequence. We previously demonstrated that a λ_{max} of 315−320 nm is indicative of lipid-exposed Trp located at the bilayer center. This conclusion was based upon direct fluorescence quenching measurements of Trp depth described in Ren et al. (*16*). We also previously identified two different cases in which a Trp at the center of the hydrophobic core of a polyLeu peptide gave rise to more red-shifted spectra. One was when the Trp moved to the bilayer surface, and the other was when the Trp remained deeply located in the bilayer but the peptides oligomerized. In the former case, the red shift is due to the more polar environment near the membrane surface and, in the latter case, presumably reflects the increase in local polarity around Trp upon its shift from an environment composed of lipid hydrocarbon to one in which it is adjacent to other peptides, and their polar peptide bonds (*17*).

Because of the highly hydrophobic sequences studied, oligomerization seemed to be the most likely explanation for the red-shifts observed for peptides studied in this report. To test this hypothesis, we first ruled out the possibility that the peptides were moving toward the bilayer surface by measuring the depth of the Trp depth residue for each peptide. To do this, our recently introduced dual quencher assay was used (*23*).[2] In this assay the quenching by an aqueous quencher molecule (acrylamide) is compared to that induced by a quencher that locates deeply within the lipid bilayer (10-doxyl nonadecane, 10-DN). A Trp close to the bilayer surface is quenched strongly by acrylamide, whereas one close to the bilayer center is quenched strongly by 10-DN. The ratio of quenching by acrylamide to that by 10-DN (Q-ratio, see Experimental Procedures) is related to Trp depth (*23*). The validity of this assay was confirmed in our previous report using a series of peptides with Trp at different

[2] In previous studies we used parallax analysis of Trp quenching in order to measure Trp depth (*4*, *16*, *17*). Parallax analysis was not used for the peptides in this study because of complications that can occur when lipid headgroup composition is varied and because of the relative ease of the dual quencher method (*18*).

Table 1: Trp Fluorescence Quenching of the K_2-Flanked, DK-Clanked, and the KD_3-Flanked Peptides by Acrylamide and 10-Doxyl Nonadecane

peptide(s)[a]	lipid[a]	pH	F_o/F^b acrylamide	F_o/F^b 10-DN	Q-ratio[b]
K_2	DOPC	7.6	1.23 ± 0.1	2.74 ± 0.03	0.133 ± 0.05
KD_3	DOPC	4	1.03 ± 0.01	2.82 ± 0.47	0.014 ± 0.01
KD_3	DOPC	7.6	1.05 ± 0.06	1.69 ± 0.24	0.067 ± 0.09
KD_3	20% DOPG	4	1.03 ± 0.03	2.54 ± 0.33	0.017 ± 0.01
KD_3	20% DOPG	7.6	1.02 ± 0.02	1.32 ± 0.17	0.052 ± 0.098
K_2	20% DOPG	7.6	1.14 ± 0.02	2.95 ± 0.07	0.074 ± 0.01
$KD_3 + K_2$	DOPC	7.6	1.03 ± 0.03	1.47 ± 0.06	0.056 ± 0.068
DK	DOPC	7.6	1.09 ± 0.02	1.95 ± 0.07	0.089 ± 0.03

[a] Samples contained 2 μM peptide in 200 μM lipid vesicles. [b] See experimental procedures for definition of F_o/F and Q-ratio. Average values and standard deviations for triplicates are shown. Acrylamide was used at 190 mM, not 235 mM as in ref *23*.

depths (*23*). It was shown that the Q-ratio for a Trp at the bilayer center is 0−0.2, whereas the ratio for a Trp at the polar/nonpolar boundary is close to 1.4. The dependence of Q-ratio on Trp depth was found to be nearly linearly dependent on Trp depth between these extremes (*23*).

Table 1 shows the results of quenching studies on the KD_3-, DK-, and K_2-flanked peptides. In 100% DOPC vesicles at both neutral and low pH, the quenching of the Trp at the center of the hydrophobic sequence gave very low Q-ratios (<0.2) for all three peptides. The K_2- and KD_3-flanked peptides were also examined in 20% DOPG-containing vesicles and were found to exhibit similarly low Q-ratios. This shows that the Trp residues locate at the bilayer center in all cases and rules out the possibility that the red-shifted λ_{max} is due to movement toward the bilayer surface.

Interpretation of the pH Dependence of λ_{max} in Terms of Peptide Behavior. Effect of Varying the Concentration of Peptide Within the Lipid Bilayers Upon Emission. Since Trp depth does not explain the red shifts of the KD_3- and DK-flanked peptides at neutral pH, peptide oligomerization within the bilayer seemed to be the most likely explanation for the red shifts in fluorescence. It has been demonstrated previously that red shifts can reflect oligomerization by experiments in which red shifts were abolished by diluting the concentration of peptides in vesicles (*17*) and by experiments showing red shifts can be induced by locally increasing the concentration of a peptide within a bilayer in situ. This was done by incorporating a peptide that is excluded from gel phase domains into a fluid lipid bilayer and then inducing partial gel phase formation by decreasing temperature to below the phase transition temperature (G. A. Caputo and E. London, unpublished observations).

To confirm that oligomers are present in the samples of KD_3- and DK-flanked peptides and that oligomerization induced a red shift, Trp λ_{max} was studied as the lipid:peptide ratio was varied. Weakly associated oligomers should dissociate and result in a Trp blue-shift upon dilution of peptide in the bilayer (i.e., an increased lipid:peptide ratio).

As shown in Figure 4, at neutral pH Trp λ_{max} for the KD_3-flanked peptide exhibited a significant concentration dependence in DOPC vesicles, decreasing 6 nm as lipid concentration was increased from 200 to 1500 μM at constant peptide concentration, while the λ_{max} for the DK-flanked peptide decreased by 4 nm over the same range. In contrast, Trp λ_{max} for the K_2-flanked peptide showed only a very weak concentration dependence, decreasing by only 1 nm when lipid concentration was increased from 200 to 1500 μM.

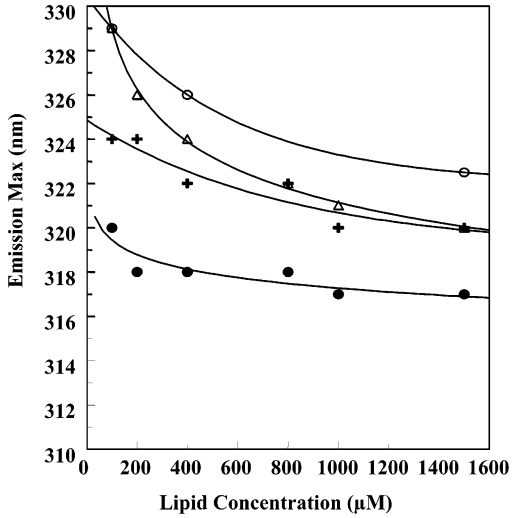

FIGURE 4: Trp emission maximum of K_2-flanked, DK-flanked, and KD_3-flanked peptides vs DOPC concentration. (•) K_2-flanked, (+) DK-flanked, (△) KD_3-flanked, and (○) K_2-flanked plus KD_3-flanked. Samples contained 2 μM total peptide incorporated into DOPC vesicles dispersed in 10 mM sodium phosphate, 150 mM NaCl, pH 7.6. Average values from duplicate experiments are shown. λ_{max} values were reproducible to within ±1 nm.

These results strongly suggest that both the KD_3- and DK-flanked peptides oligomerize in DOPC vesicles at neutral pH and that their level of oligomerization is reduced by their dilution within lipid bilayers. Because the fraction of buried Trp residue buried within an oligomer should increase with increasing oligomer size, it appears likely that the KD_3-flanked peptides oligomerize more than the DK-flanked peptide and that the K_2-flanked peptide may have, at most, only a very low degree of oligomerization under these conditions.

A similar experiment was performed for the KD_3-flanked peptide in vesicles containing 20% DOPG vesicles at neutral pH. Trp λ_{max} decreased from 331 nm at 200 μM lipid to 324 nm at 1500 μM at lipid, again consistent with oligomerization that decreases upon peptide dilution in the lipid bilayer (data not shown).

It should be noted that oligomerization of the KD_3- and DK-flanked peptides is also consistent with the reduced level of quenching of their Trp residues by 10-DN. In oligomers, Trp would tend to become buried within the oligomer interior, and thus be less exposed to lipid. As a result, Trp accessibility to, and quenching by, 10-DN should be sterically decreased. Consistent with this prediction, inspection of Table 1 shows that quenching by 10-DN was significantly de-

Table 2: Trp Emission λ_{max} for K_2-Flanked, DK-Flanked, and KD_3-Flanked Peptides Incorporated into DOPC Vesicles Individually and in Various Combinations at pH 7.6[a]

peptides flanked by	[lipid]:[total peptide]	Trp λ_{max}
K_2	200:1	318
KD_3	200:1	323
$KD_3 + K_2$	200:1	325.5
KD_3/ K_2	200:1	319
K_2	200:2	318
KD_3	200:2	324.7
$KD_3 + K_2$	200:2	326
KD_3/ K_2	200:2	320
K_2	200:1	318
DK	200:1	322
DK+ K_2	200:2	320
DK/ K_2	200:1	320

[a] Combinations denoted with a "+" indicate peptides mixed within a single population of vesicles. Combinations denoted with a "/" indicate mixtures of two vesicle populations, each of which contains a different type of peptide. Average values for duplicates are shown. The λ_{max} values were generally found to be consistent within ±1 nm.

creased for the DK- and KD_3-flanked peptides under conditions (neutral pH) in which they exhibit red shifted λ_{max} (i.e., oligomerize), whereas the K_2-flanked peptide remained strongly quenched by 10-DN.[3]

Interaction Between the K_2-Flanked and the KD_3-Flanked Peptides in DOPC Vesicles. To determine whether electrostatic attractions between residues at helix-flanking position could influence helix−helix interactions, mixtures of K_2-flanked and KD_3-flanked peptides were incorporated into DOPC vesicles. At neutral pH, these peptides might attract each other because the KD_3-flanked peptide should have a net negative charge whereas the K_2-flanked peptide should have a net positive charge.

To assess helix−helix interaction, Trp emission λ_{max} was compared in samples in which K_2- and KD_3-flanked peptides were in separate vesicle populations to that in samples in which they were incorporated into a single population (Table 2). At neutral pH, the K_2- and the KD_3-flanked peptides at a 200:1 lipid:peptide ratio in separate vesicles gave λ_{max} values of 318 and 323 nm, respectively. When equal volumes of these samples were mixed, an average λ_{max} value of 319 nm was obtained. (This value is not exactly midway between the individual values because the fluorescence of the K_2-flanked peptide is stronger than that of the KD_3-flanked peptide in these samples.) Obviously, there was no interaction possible between the two peptides in this mixture because they were not incorporated in the same vesicle. These values were compared to those obtained at neutral pH when both peptides were incorporated into a single population of vesicles at a 1:1 ratio, such that total lipid:peptide ratio was 200:2. The λ_{max} for the combination of peptides in the same vesicles was 326 nm. Since if there had been no interaction Trp λ_{max} should have been 319 nm, this result strongly suggests that there is an interaction between K_2- and KD_3-flanked peptides when they are in the same vesicle. Assuming that the degree of red shift is related to the degree of peptide-peptide interaction, it is noteworthy that the λ_{max} with both peptides in the same vesicle at a total lipid:peptide ratio of 200:2 (326 nm) was more slightly more red-shifted than that for the KD_3-flanked peptide by itself at a lipid:peptide ratio of 200:2 (324.7) and significantly more red-shifted than for K_2-flanked peptide by itself at a lipid:peptide ratio of 200:2 (318 nm). This indicates that the K_2- and KD_3-flanked peptides interact with each other to a greater extent than they interact with themselves. This conclusion is reinforced by comparison of Trp λ_{max} (325.5 nm) in a mixture of K_2- and KD_3-flanked peptides in the same population of vesicles to that they have by themselves at a total lipid:peptide ratio decreased to 200:1 (Table 2).

One alternate, although unlikely, explanation for the red shift is that the mixture of KD_3- and K_2-flanked peptides move toward the membrane surface, so that the Trp located in a more shallow polar location. To eliminate this possibility, Trp depth in samples containing mixtures of the KD_3- and K_2-flanked peptides was measured using the dual quencher analysis. As shown in Table 1, the Q-ratio remains low, showing Trp residues do not move toward the surface.

To confirm the presence of oligomerization in vesicles containing a mixture of K_2- and KD_3-flanked peptides, the effect of varying the concentration of peptides within the bilayer upon λ_{max} was determined. As shown in Figure 4, Trp λ_{max} decreased by 6 nm as the lipid concentration was increased from 100 to 1500 μM. This is consistent with oligomerization that decreases upon dilution of peptides within the bilayer.

As might be expected the interaction between these two peptides was dependent upon pH. At pH 2, each peptide by itself and mixed in a single population of DOPC vesicles gave an emission maximum of 319 nm (data not shown). This suggests the K_2- and KD_3-flanked peptides do not interact at pH 2. This is as expected because Asp and Lys residues on the KD_3-flanked peptide should be protonated at pH 2, so that both the KD_3- and K_2-flanked peptides would have a positive charge, and repel each other. Combined with the results above, these experiments indicate that interaction at neutral pH is due to electrostatic attractions.

In another set of samples either KD_3-flanked peptide or a mixture of KD_3-flanked and K_2-flanked peptides were incorporated into vesicles prepared at pH 2.8, and then the pH was increased to 7.1. In both cases, these samples gave blue shifted fluorescence with a λ_{max} at 321 nm at low pH, which increased to 325 and 327−328 nm at neutral pH for the KD_3-flanked peptide and mixture, respectively (data not shown). This shows that the interaction between the KD_3-flanked and K_2-flanked peptides is not affected by the pH at which the samples are prepared or by the charge on the peptides at the time of their incorporation into bilayers.

Lack of Interaction Between K_2-Flanked Peptide and the DK-Flanked Peptides in DOPC SUV. As a control to confirm the influence of electrostatic attractions upon helix−helix interaction, mixtures of the DK- and K_2-flanked peptides

[3] Any steric reduction of acrylamide quenching upon oligomerization would be difficult to detect because acrylamide quenching of the deep Trp residues in these peptides is weak. In addition, it is not clear that the lateral occlusion of Trp that would occur upon oligomerization should affect acrylamide quenching, which may only respond to the distance of Trp from the bilayer surface. If this is the case, then Q-ratio should be influenced by oligomer formation as well as depth. However, an effect of oligomerization on Q-ratio would be weak for a deeply located Trp.

were studied. As shown in Table 2, the Trp λ_{max} of the mixture of the K_2- and DK-flanked peptides in a single vesicle population was equal to the average of their λ_{max} when in separate vesicles, indicating a lack of interaction between the DK- and K_2-flanked peptides at neutral pH. Since the DK-flanked peptide has no net charge at pH 7 it is presumably unable to electrostatically attract K_2-flanked peptide.

Reversibility of Red Shifts Induced by Increasing pH. When samples of the DK-peptide, KD_3-peptide, or mixture of KD_3- and K_2-flanked peptides were prepared in DOPC vesicles at pH 7 and pH was then deceased to pH 4, the red shifts observed at pH 7 were abolished. This was also seen for KD_3-flanked peptide incorporated into vesicles containing 20mol % DOPG (not shown). These results indicate that the red shifts and oligomerization observed when pH is increased are reversible when pH is decreased.

Confirming Oligomerization by DibromoTyr Quenching of Trp Fluorescence. Measurement of red shifts (when not associated with a change Trp depths) and their concentration dependence is a particularly simple method to detect oligomerization. However, it is indirect. Therefore, we performed control experiments using a very recently introduced technique in which oligomerization is detected by the quenching of Trp fluorescence in hydrophobic peptides upon their interaction with dibromoTyr-labeled peptides (*24*). This method showed that quenching and aggregation were correlated by incorporating a peptide that is excluded from gel phase domains into a fluid lipid bilayer and then inducing gel phase formation by decreasing temperature to below the phase transition temperature. Below the phase transition temperature quenching increased greatly (*24*).

In quenching experiments, oligomerization results in a decrease in Trp fluorescence in the presence of the dibromoTyr-labeled peptide (F) relative to that in the absence of the dibromoTyr-labeled peptide (Fo). Figure 5 shows that at neutral pH a K_2-flanked dibromoTyr-labeled peptide interacts much more strongly with the KD_3-flanked peptide than with the Trp-containing K_2-flanked peptide (i.e., F/F_o values are much lower in the former case). In addition, Figure 5 shows that the interaction between the dibromoTyr labeled K_2-flanked peptide and the KD_3-flanked peptides is stronger at neutral pH than it is at low pH. The observation that the decrease in quenching at low pH was reversed when pH in the sample was increased to pH 7 confirms that the pH-induced changes in quenching represent an equilibrium. These results all agree with the above-described conclusions based upon red shifts in Trp fluorescence.

Circular Dichroism of the DK-Flanked and KD_3-Flanked Peptides in DOPC SUV. Previous circular dichroism (CD) experiments indicated that the K_2-flanked peptide is highly α-helical over a wide range of pH (*4*). To determine the secondary structure of the KD_3- and DK-flanked peptides, their CD spectra were measured. For this experiment, peptides were incorporated into DOPC vesicles at pH 7. As expected, the spectra of both the KD_3- and DK-flanked peptides displayed the characteristic minima of an α-helix at 208 and 222 nm (not shown), with estimated helix contents, 71% and 80%, respectively, very similar to that of the K_2-flanked peptide (76%) (*16*).

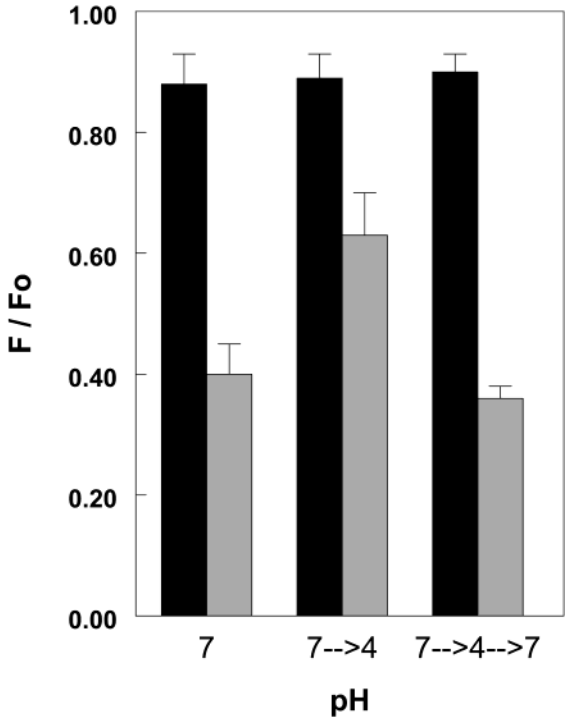

FIGURE 5: Assay of peptide-peptide interactions using quenching by dibromoTyr-labeled peptide. The quenching of Trp fluorescence by dibromoTyr is illustrated for samples containing mixtures of (black bars) K_2-flanked and dibromoTyr K_2-flanked peptides, or mixtures of (gray bars) KD_3-flanked and dibromoTyr K_2-flanked peptides. The *Y*-axis shows the ratio of Trp fluorescence intensity in samples containing 2 μM Trp-containing K_2- or KD_3-flanked peptides mixed with 1 μM dibromoTyr peptide to that in samples containing just 2 μM of the Trp-containing K_2- or KD_3-flanked peptides. Peptides were incorporated in lipid vesicles containing DOPC at a concentration of 200 μM lipid dispersed in 10 mM sodium phosphate, 150 mM NaCl, pH 6.9. After fluorescence was measured, pH was decreased to 3.8, and fluorescence was remeasured. Finally, pH was reversed to close to pH 7 with a NaOH solution and fluorescence remeasured again.

DISCUSSION

Dependence of the Behavior of Transmembrane Helices upon Flanking Residue Charge. This study shows that ionizable residues located at the positions flanking the hydrophobic core of TM helices can significantly influence helix−helix interactions. The pH dependence of the apparent oligomeric states of the peptides studies in this report is summarized schematically in Figure 6. The lowest degree of oligomerization was detected at low pH, where all the peptides should have protonated Asp and Lys residues. At low pH the K_2-flanked peptide should have a net positive charge of +4, and the DK- and KD_3-flanked peptides a charge of +2. Thus, it appears that a charge of +2 is sufficient to prevent extensive helix oligomerization. As pH approaches neutral values and Asp residues deprotonate, the DK and KD_3-flanked peptides show increased oligomerization. For the DK-flanked peptide, the Asp ionization results in a zwitterionic species, and a loss of electrostatic repulsion between peptides could explain the increased oligomerization. The same should be true for the KD_3-flanked peptide at a pH at which there is only partial Asp ionization, i.e., near pH 6−7, where maximum red shift is observed. However, there is clearly an additional factor influencing the oligomerization of the KD_3-peptide because (1) it shows

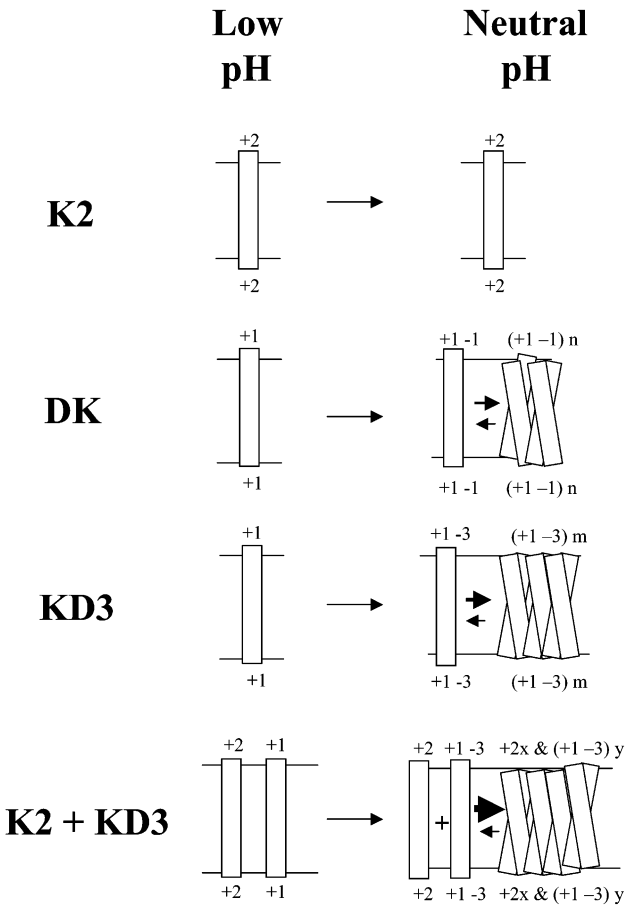

FIGURE 6: Schematic illustration of bilayer-inserted peptide behavior under different conditions. The identity of the flanking residues is shown on the left. Peptides are illustrated by rectangles with the charge at low and near neutral pH shown at each end. The coefficients *n*, *m*, *x*, and *y* represent the unknown stoichiometries of the oligomers. Although changes in oligomerization state are shown as if they involve both changes in oligomer size and shifts in the monomer/oligomer equilibrium, changes may be occurring in only one of these parameters. In addition, it is uncertain whether monomers or a small oligomer are present under conditions (e.g., low pH) giving the least oligomerization.

a larger red shift than the DK-peptide and (2) its fluorescence remains red-shifted above pH 6−7, pH values at which it should have a net negative charge. These results suggest that there is some interaction involving ionized Asp residues that more than compensates for any electrostatic repulsions. Interactions between the charged Lys residues on one KD_3-flanked peptide and the Asp residues on a second KD_3-flanked peptides may be involved, but the fact that the red shifts for both the KD_3- and DK-flanked peptides persist somewhat at very high pH values, where flanking Lys residues should be partly or fully deprotonated, suggests other factors that we do not understand are involved. Interactions with trace amounts of metal ions are a possibility, but addition of 5 mM EDTA had no effect upon the emission of the KD_3-flanked peptide at pH 7.

pK_a Values for Flanking Asp and Lys Residues Close to the Bilayer Surface. As noted in the Results, the apparent pK_a values observed for the flanking Asp and Lys residues can be estimated from fluorescence. Relatively unambiguous values can be estimated for the DK- and K_2-flanked peptides, which have the fewest Asp and Lys residues. Inspection of the pH titration data shows there are inflection points in fluorescence intensity at 330 nm for these peptides in DOPC vesicles that yield apparent pK_a values for flanking Asp and Lys of 5.5−6 and 9.5−10, respectively.[4] These values are shifted about 0.5−1.5 pH unit from what would be expected for these residues in aqueous solution (4.5 and 10.4, respectively) (*25*). Such shifts are about what would be estimated given the energetic difference (about 1 kcal/mol) between locating a charged Asp or Lys group in the polar/interfacial part of the bilayer relative to that for a similar group in the uncharged state (*5*). (In the case of the DK peptide, there may be an additional effect on pK_a due to the effect of neighboring charged Asp and Lys residues on each other.)

Effect of Anionic Lipid on Helix−Helix Interaction. As noted in the Results, DOPG-induced Asp and Lys pK_a shifts are readily explained by the surface charge on vesicles containing DOPG. In addition to pK_a shifts, it was found that near neutral pH the KD_3-flanked peptide gave more red-shifted fluorescence in vesicles containing 20% DOPG than in 100% DOPC vesicles. Assuming this increased red shift reflects increased oligomerization, electrostatic repulsion between negatively charged DOPG and negatively charged KD_3-flanked peptide can explain this behavior. This repulsion would raise the free energy of a monomeric state in which anionic KD_3-flanked helices are surrounded by anionic lipid, relative to a more highly oligomeric state in which there would be less anionic lipid in the immediate vicinity of each helix. In other words, lipid−peptide repulsion would be a driving force for helix−helix interaction.

In contrast to its affects on the KD_3-flanked peptide, 20 mol % DOPG had little, if any affect on the degree of red shift for the DK- and K_2-flanked peptides at neutral pH. The DK-flanked peptide is zwitterionic near neutral pH, and its self-association should not be affected significantly by lipid charge. In the case of the K_2-flanked peptide, it would be expected that attractions between the Lys and anionic lipid at neutral pH would antagonize oligomerization. However, since the K_2 peptide is already monomeric in vesicles containing 100% DOPC (or in the form of a very small oligomer in which its Trp are exposed to lipid) no detectable change in oligomerization would be expected in the presence of DOPG.

Interactions Between Helices Having Different Flanking Ionizable Residues. This report also shows charge can affect the interactions between helices with differing flanking residues. Mixtures of K_2-flanked and KD_3- flanked peptides in DOPC vesicles tended to associate at pH 7.6. Under these conditions, the K_2-flanked peptide should have a net positive charge, and the KD_3-flanked peptide should have a net negative charge. In contrast, no interaction was observed for the cationic K_2-flanked peptide and zwitterionic DK-flanked peptide or for the K_2-flanked and KD_3-flanked peptides at low pH, where they would both be cationic. Therefore, it appears electrostatic attractions between oppositely charged groups at helix-flanking positions can be sufficient to promote helix−helix interactions in bilayers.

Comparison to Other Studies and the Effect of the Depth of an Ionizable Residue Within the Bilayer Upon Its

[4] It should be noted that λ_{max} and F350/330 ratio give weighted averages, whereas fluorescence intensity at a single wavelength is linear in the amount of species present in a mixture. As a consequence, the latter parameter allows more accurate estimation of pK_a values.

Behavior. Cosson and Bonifacino found interactions between an interfacial Glu residue on the α subunit and an interfacial Lys residue on the β subunits of the transmembrane domains of the Class II major histocompatibility complex protein contribute to transmembrane helix−helix interactions (*10*). In a study using the glycosylation mapping technique, Chin and von Heijne (*3*) found evidence for an interaction between an Asp residue placed close to the edge of a hydrophobic segment of a helix with a Lys residue on the same side of the helix but one turn more distant from the bilayer surface.

The interpretation of such studies is complicated by a lack of information on the ionization state of the residues involved. This ambiguity partly arises from the unknown dependence of the pK_a, and thus charge, of ionizable residues upon their depth in a bilayer. Previous studies indicate that deeply buried residues are not likely to be charged (*4*, *26*). On the other hand, ionizable residues deep within a bilayer, but exposed to a local polar environment such as an aqueous pore or polar region of a protein, could exist in a charged form. Salt bridges in the in the LHC−II protein of the light-harvesting complex from plants may fall into this category (*27*). The influence of electrostatic interactions between acidic and basic residues is less ambiguous in our experiments because of the ability to identify ionization states by varying pH, which reveals when the flanking Asp and Lys groups are charged or uncharged.

Implications for Regulation of Helix−Helix Interactions in Biological Membranes. The interactions observed in this report suggest that electrostatic interactions between helix-flanking residues may be large enough to play an important role in modulating helix−helix interaction and membrane protein folding in natural membranes. Lipid charge could also regulate helix−helix interaction in vivo. If a protein is exposed to environments with different amounts of anionic lipid as it moves from one compartment of a eukaryotic membrane to another (e.g., from endoplasmic reticulum to plasma membrane), it could undergo changes in how tightly its transmembrane helices associate with the helices of other membrane proteins, or how tightly its own helices are packed. It should also be noted that pH may be an important factor in regulating helix−helix interaction in vivo via its affect on ionizable residue charge. The low pH in the lumen of endocytic vacuoles of eukaryotic cells should be sufficient to at least partially protonate helix-flanking Asp and Glu residues, as well as fully protonate His residues.

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