Cumulative Effects of Amino Acid Substitutions and Hydrophobic Mismatch upon the Transmembrane Stability and Conformation of Hydrophobic α-Helices†

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ABSTRACT: The effects of amino acid substitutions upon the behavior of poly(Leu)-rich α-helices inserted into model membrane vesicles were investigated. One or two consecutive Leu residues in the hydrophobic core of the helix were substituted with A, F, G, S, D, K, H, P, GG, SS, PG, PP, KK, or DD residues. A Trp placed at the center of the sequence allowed assessment of peptide behavior via fluorescence emission λ_{max} and dual quenching analysis of Trp depth [Caputo, G. A., and London, E. (2003) *Biochemistry 42*, 3265−3274]. In vesicles composed of dioleoylphosphatidylcholine (DOPC), all of the peptides with single substitutions adopted a transmembrane (TM) state. Experiments were also performed in thicker bilayers composed of dierucoylphosphatidylcholine (DEuPC). In DEuPC vesicles TM states were destabilized by mismatch between helix length and bilayer thickness. Nevertheless, in DEuPC vesicles TM states were still prevalent for peptides with single substitutions, although less so for peptides with P, K, H, or D substitutions. In contrast to single substitutions, certain consecutive double substitutions strongly interfered with formation of TM states. In both DOPC and DEuPC vesicles DD and KK substitutions abolished the normal TM state, but GG and SS substitutions had little effect. In even wider bilayers, a SS substitution reduced the formation of a TM state. A peptide with a PP substitution maintained the TM state in DOPC vesicles, but in DEuPC vesicles the level of formation of the TM state was significantly reduced. Upon disruption of normal TM insertion peptides moved close to the bilayer surface, with the exception of the KK-substituted peptide in DOPC vesicles, which formed a truncated TM segment. These studies begin to provide a detailed relationship between sequence and the stability of TM insertion and show that the influence of insertion-destabilizing residues upon hydrophobic helices can be strongly modulated by properties such as mismatch. For certain helix-forming hydrophobic sequences, sensitivity to lipid structure may be sufficient to induce large conformational changes in vivo.

The transmembrane (TM) segments of membrane proteins are commonly composed of hydrophobic α-helices 15−25 amino acids in length. Primary sequence is one major factor determining whether a helix will be transmembranous, as well as how it interacts with other helices within membranes (*4*, *5*). Polypeptides composed of hydrophobic residues have a strong tendency to form TM structures because of the favorable energetics for the loss of exposure of hydrophobic side chains to water. Polar and ionizable residues have a relatively low abundance in the core of TM segments, presumably because the unfavorable energetics of their exposure to a nonpolar environment destabilizes TM insertion (*5*). Pro, which should destabilize TM insertion by leaving an unsatisfied backbone H-bond, is also of relatively low abundance in TM segments.

Nevertheless, all of these insertion-destabilizing residues are found in TM sequences to some degree and play important roles in membrane protein structure and function. For example, recent studies have confirmed the importance of polar residues in TM helix association (*6*−*9*). Pro residues may be functionally important in membrane helices because they can introduce a kink of about 10−20 degrees and/or because they create a more flexible overall structure (*10*, *11*). Polar amino acids are believed to reduce the energetic cost of their insertion into membranes by forming hydrogen bonds (*9*). Ionizable residues can reduce the energetic cost of burial within a bilayer by existing in an uncharged state or by forming salt bridges (*12*−*14*).

In addition to sequence, the behavior of a membrane-inserted helix can be influenced by hydrophobic mismatch, which is the difference between the length of the hydrophobic segment of a membrane-inserting polypeptide and the width of the hydrophobic core of the membrane bilayer. TM helices can respond to mismatch in a number of ways that minimize the contact between hydrophobic and nonhydrophobic surfaces (*15*). If membrane width is greater than the length of a hydrophobic helix (i.e., the hydrophobic segment is too short to transverse the membrane, called negative mismatch), the helix may aggregate within the membrane, move to the membrane surface, or remain in aqueous solution (*3*, *15*−*17*). If membrane width is less than the length of a hydrophobic helix (i.e., called positive mismatch), a helix may bend or adopt a tilted structure in order to bury as much

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hydrophobic surface area in the bilayer as possible. Alternately, it may protrude slightly from the bilayer and/or form TM oligomers (*15*, *16*, *18*, *19*).

Despite such information, our ability to predict the behavior of membrane-inserted sequences is fragmentary. Simple hydropathy parameters, based on the hydrophobicity of individual amino acids, ignore lipid bilayer properties, the effect of the position of insertion-destabilizing residues within a hydrophobic sequence, and nonadditive combinatorial effects of multiple destabilizing residues. Indications that these latter two factors influence the behavior of hydrophobic sequences come from studies by von Heijne and colleagues using glycosylation mapping (*20*, *21*). This method tracks the position of TM helices by identifying the residue in a hydrophobic sequence that is positioned at the lumenal edge of the endoplasmic reticulum membrane during protein biosynthesis (*22*). Their studies show that the presence of an insertion-destabilizing residue can perturb the position of a helix within a membrane (*20*, *21*). They also find that, at least when close to one end of TM helix, interactions between acidic and basic residues can cancel out the tendency of a single isolated ionizable residue in a TM sequence to shift helix position (*14*).

Glycosylation mapping has important advantages, but it does not reveal if non-TM orientations are present or allow wide variation of conditions such as pH and lipid composition. One alternative is to study the behavior of synthetic hydrophobic helices (*3*, *18*, *23*, *24*). We have used Lys-flanked poly(Leu) peptides containing a hydrophobic core with a Trp at the center of the sequence. These peptides form α-helices when incorporated into model membrane vesicles (*3*, *23*). Their Trp fluorescence can be used to monitor both peptide location and self-association within bilayers (*3*, *23*). Using this approach, we found that mismatch can significantly alter the degree to which hydrophobic peptides take on a TM orientation and/or oligomerize within model membranes (*23*). Introduction of a single polar or ionizable residue into the core of these peptides did not prevent their formation of a TM structure within dioleoylphosphatidylcholine (DOPC)[1] bilayers (*12*).

In the present study poly(Leu) peptides with a wide range of amino acid substitutions were examined under conditions in which the degree of mismatch was varied. This allowed a detailed investigation of how substitutions affect the stability of TM orientation. In addition to novel information about the sequence dependence of TM insertion stability, it was found that sequence and mismatch can interact to modulate the stability of the TM state. The possibility that lipid properties influence hydrophobic helix conformation in vivo is discussed.

[1] Abbreviations: 10-DN, 10-doxylnonadecane; DMoPC, diC14:1Δ9cPC; DPoPC, diC16:1Δ9cPC; DOPC, diC18:1Δ9cPC; DEiPC, diC20:1Δ11cPC; DEuPC, diC22:1Δ13cPC; DNPC, diC24:1Δ15cPC; DQA, dual quencher analysis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; pL(L11) = pL(W13), $K_2GL_9WL_9K_2A$; pL(A11), $K_2GL_7ALWL_9K_2A$; pL(F11), $K_2GL_7FLWL_9K_2A$; pL(S11), $K_2GL_7SLWL_9K_2A$; pL(H11), $K_2GL_7HLWL_9K_2A$; pL(K11), $K_2GL_7KLWL_9K_2A$; pL(D11), $K_2GL_7DLWL_9K_2A$; pL(G11), $K_2GL_7GLWL_9K_2A$; pL(P11), $K_2GL_7PLWL_9K_2A$; pL(P10G11), $K_2GL_6PGLWL_9K_2A$; pL(P10P11), $K_2GL_6PPLWL_9K_2A$; pL(D10D11), $K_2GL_6DDLWL_9K_2A$; pL(K10K11), $K_2GL_6KKLWL_9K_2A$; pL(G10G11), $K_2GL_6GGLWL_9K_2A$; pL(S10S11), $K_2GL_6SSLWL_9K_2A$; pL(W4), $K_2CWL_9AL_9L_2A$; PC, phosphatidylcholine.

EXPERIMENTAL PROCEDURES

Materials. Peptides $K_2GL_9WL_9K_2A$ [pL(L11) = pL(W13)], $K_2GL_7ALWL_9K_2A$ [pL(A11)], $K_2GL_7FLWL_9K_2A$ [pL(F11)], $K_2GL_7SLWL_9K_2A$ [pL(S11)], $K_2GL_7HLWL_9K_2A$ [pL(H11)], $K_2GL_7KLWL_9K_2A$ [pL(K11)], $K_2GL_7DLWL_9K_2A$ [pL(D11)], $K_2GL_7GLWL_9K_2A$ [pL(G11)], $K_2GL_7PLWL_9K_2A$ [pL(P11)], $K_2GL_6PGLWL_9K_2A$ [pL(P10G11)], $K_2GL_6PPLWL_9K_2A$ [pL(P10P11)], $K_2GL_6DDLWL_9K_2A$ [(pL(D10D11)], $K_2GL_6KKLWL_9K_2A$ [pL(K10K11)], $K_2GL_6GGLWL_9K_2A$ [pL(G10G11)], $K_2GL_6SSLWL_9K_2A$ [pL(S10S11)], and $K_2CWL_9AL_9L_2A$ [pL(W4)] were purchased from Research Genetics (ResGen Division of Invitrogen) (Huntsville, AL). The N-termini of all of the peptides were acetylated, and the C-termini of all of the peptides were amide-blocked. A series of 1,2-diacyl-*sn*-glycero-3-phosphocholines (phosphatidylcholines, PCs) including diC14:1Δ9cPC (dimyristoleoyl-PC, DMoPC), diC16:1Δ9cPC (dipalmitoleoyl-PC, DPoPC), diC18:1Δ9cPC (dioleoyl-PC, DOPC), diC20:1Δ11cPC (dieicosenoyl-PC, DEiPC), diC22:1Δ13cPC (dierucoyl-PC, DEuPC), and diC24:1Δ15cPC (dinervonoyl-PC, DNPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Concentrations of lipids purchased as solutions dissolved in chloroform were confirmed by dry weight. Stock solutions of lipids dissolved in chloroform were stored at −20 °C. Lipid purity was confirmed by TLC on silica gel plates chromatographed in 65:25:4 chloroform/methanol/water (v/v). Acrylamide was purchased from Sigma Chemical Co. (St. Louis, MO). 10-Doxylnonadecane (10-DN) was purchased from Aldrich Chemical Co. (Milwaukee, WI). (Discontinued; contact authors for availability.) It was stored as a 27−29 mM stock solution in ethanol at −20 °C.

Peptides were purified via reverse-phase HPLC using a C18 column with an 2-propanol/water/0.5% v/v trifluoroacetic acid mobile phase as described previously (*25*). Peptides containing consecutive hydrophilic substitutions were often soluble in 20−35% 2-propanol as opposed to the 40% that was needed for more hydrophobic peptides with either no or one hydrophilic substitution. As a result, gradients were started at a lower 2-propanol concentration for the more hydrophilic peptides. Peptide purity was confirmed using MALDI-TOF mass spectrometry (CASM, SUNY, Stony Brook). Final purity was on the order of 90% or better. In general, the only significant impurities appeared to be peptides with single amino acid deletions, which likely behave very similarly to the full-length peptides. Peptide concentrations were measured by absorbance spectroscopy on a Beckman DU-650 spectrophotometer using an extinction coefficient of 5560 M^{-1} cm^{-1} at 280 nm. Peptides were stored in ethanol or 1:1 2-propanol/water (v/v) at 4 °C.

Sample Preparation. Model membrane vesicles were prepared using the ethanol dilution method (*3*, *23*). Peptides dissolved in ethanol or 1:1 2-propanol/water and lipid dissolved in chloroform were mixed and then dried under a stream of N_2. Samples were then dried under high vacuum for 1 h. After 10 μL of 100% ethanol was added to dissolve the samples, 790 μL of PBS (100 mM $NaPO_4$ and 150 mM NaCl, pH 7.1−7.3) was added while vortexing to disperse the lipid−peptide mixture. Unless otherwise specified, final concentrations were 2 μM peptide and 500 μM lipid.

Fluorescence Measurements. Fluorescence data were obtained on a SPEX τ2 fluorolog spectrofluorometer operat-

ing in the steady-state mode at room temperature. Measurements were made in semimicro quartz cuvettes (1 cm excitation path length and 4 mm emission path length) using a 2.5 mm excitation slit width and 5 mm emission slit width (bandwidths of 4.5 and 9 nm, respectively). The excitation wavelength used to excite Trp was 280 nm unless otherwise noted. Fluorescence emission spectra were measured over the range 300−375 nm. Fluorescence from background samples containing lipid but no peptide was subtracted from reported values.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were recorded on a Jasco J-715 CD spectrophotometer at room temperature using a 1 mm path length quartz cuvette. Samples were prepared as noted above with the exception that PBS diluted 10-fold with water was used to reduce solvent interference at wavelengths below 200 nm. Control experiments using undiluted PBS showed similar spectral shapes but, in some cases, slightly stronger ellipticity (up to a 20% increase). Spectra were the average of 150 scans. Backgrounds from samples lacking peptide were subtracted. Overall α-helix content was analyzed using three deconvolution programs: SELCON3 (*26*), CONTINLL (*27*), and CDSSTR (*28*).

Acrylamide Quenching Measurements. To measure acrylamide quenching, the fluorescence of 800 μL samples containing model membrane-incorporated peptides or background samples was determined both before and after the addition of a 50 μL aliquot of acrylamide from a 4 M stock solution dissolved in water. Fluorescence was measured at an excitation wavelength of 295 nm, to reduce acrylamide absorbance (and the resulting inner filter effect), and emission at a wavelength of 340 nm, to eliminate interference from the Raman band of water. Inner filter corrections for acrylamide absorbance were made as previously described (*29*).

10-Doxylnonadecane Quenching Measurements. To measure the efficiency of 10-doxylnonadecane (10-DN) quenching, model membrane-incorporated peptides or backgrounds lacking were prepared as described above except that in quencher-containing samples either 10 mol % (for DOPC vesicles) or 12 mol % (for DEuPC vesicles) of the lipid was replaced by equimolar 10-DN. [The larger fraction of quencher used in the DEuPC samples maintained a constant ratio of doxyl groups to hydrophobic volume, which is roughly 20% larger for DEuPC, which has C22:1 acyl chains, than it is for DOPC, which has C18:1 chains (*3*).] Fluorescence excitation and emission were measured at 280 and 330 nm, respectively.

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 determine Trp depth in lipid bilayers. The *Q*-ratio was calculated from the formula *Q*-ratio $= [(F_o/F_{acrylamide}) - 1]/[(F_o/F_{10-DN}) - 1]$, where F_o is the fluorescence of a sample lacking quencher and $F_{acrylamide}$ and F_{10-DN} are the fluorescence intensities in the presence of acrylamide or 10-DN, respectively.

Measurements of Quencher-Induced Shifts in Emission λ_{max}. Samples containing either no quencher, acrylamide, or 10-DN were prepared as described above. Fluorescence excitation was set at 280 nm, and emission spectra were recorded over the range 300−375 nm. The λ_{max} values were obtained after background spectra were subtracted from samples lacking peptide.

Measurements of the Effect of Decane on λ_{max} of Trp Emission. To determine the effect of decane, sequential 2 μL aliquots of decane/ethanol (1:9 v/v) were added to samples containing 2 μM peptide incorporated into 500 μM DEuPC vesicles. Samples were allowed to equilibrate for approximately 3 min after the addition of each aliquot, and then emission spectra were recorded. For the decane dilution experiments, 4 μL of 1:9 decane/ethanol was added to an 800 μL sample containing 1 μM peptide incorporated into ethanol dilution vesicles with 125 μM DEuPC. After measurement of a fluorescence emission spectrum, 160 μL of 5 mM sonicated DEuPC vesicles [prepared as described previously (*30*)] lacking peptide and decane was added. Samples were allowed to equilibrate for approximately 5 min before their emission spectra were remeasured.

RESULTS

Fluorescence λ_{max} of Peptides in Bilayers of Varying Thickness. The behavior of bilayer-inserted poly(Leu) peptides with single or consecutive double amino acid substitutions was studied. These 25-residue Lys-flanked peptides contained a Trp residue at the center of a hydrophobic 18-residue Leu sequence and could potentially form hydrophobic helices that are 27−30 Å long, sufficient to easily span the hydrophobic core of bilayers composed of DOPC, a lipid with monounsaturated 18-carbon acyl chains.

To initially characterize the effects of substitutions, the λ_{max} of Trp fluorescence emission was measured as a function of bilayer width, which was varied by preparing vesicles from lipids with monounsaturated acyl chains of various lengths. A lipid-exposed Trp residue located at the center of the hydrocarbon core of a bilayer exhibits a characteristic highly blue shifted emission, with a λ_{max} (uncorrected) in the range of 315−318 nm (*3*). As a Trp residue moves toward the more polar membrane surface, λ_{max} gradually shifts to 335−340 nm (*3*, *23*, *30*). A smaller red shift of the fluorescence for a Trp at the bilayer center can also be detected upon helix oligomerization (*23*). This shift presumably reflects the change in local environment upon replacement of contacts between Trp and lipid with contacts between Trp and polypeptide.

Figure 1A shows the effect of bilayer width on the Trp λ_{max} of poly(Leu) peptides containing the Trp at residue 13 and various single substitutions (A, G, F, P, H, K, D, and S) in place of a Leu residue at position 11. In each case, the highly blue shifted λ_{max} characteristic of a TM helix containing a lipid-exposed Trp at the bilayer center (*3*, *23*) was observed in bilayers of intermediate width (DOPC or DEiPC). More strongly red shifted fluorescence was observed both in thicker bilayers (with longer acyl chains, resulting in negative mismatch) and thinner bilayers (with shorter acyl chains that result in positive mismatch). In thicker bilayers [formed by DEuPC or 2:1 DNPC/DEuPC (mol/mol)[2]], this red shift has been proposed to involve a subpopulation of peptides forming a non-TM state in which the Trp is closer

[2] In the case of vesicles containing DNPC, it should be noted that 2:1 (mol/mol) DNPC/DEuPC mixtures were used instead of pure DNPC to avoid forming a gel-state bilayer. DNPC has a melting temperature close to room temperature (*1*).

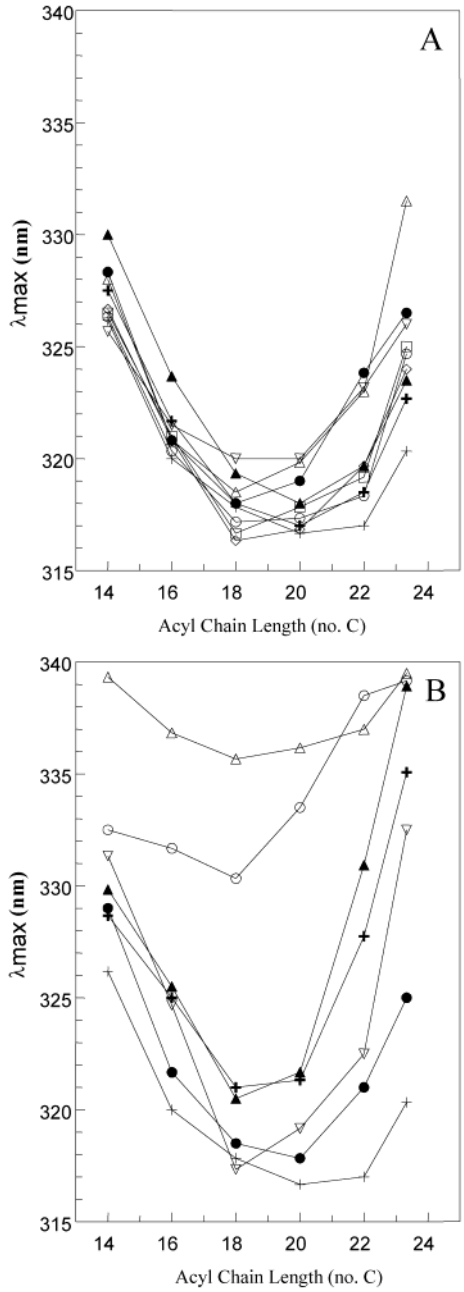

FIGURE 1: Effects of lipid acyl chain length on the Trp emission λ_{max} of peptides incorporated into lipid vesicles. (A) Peptides with a single substitution: pL(L11) (crosses), pL(P11) (open triangles), pL(S11) (open circles), pL(G11) (bold crosses), pL(D11) (filled triangles), pL(K11) (filled circles), pL(H11) (open inverted triangles), pL(A11) (open diamonds), and pL(F11) (open squares). (B) Peptides with two substitutions: pL(D10D11) (open triangles), pL(K10K11) (open circles), pL(P10P11) (filled triangles), pL(P10G11) (bold crosses), pL(G10G11) (filled circles), pL(S10S11) (open inverted triangles), and pL(L10L11) (crosses). Samples contained 2 μM peptide incorporated into vesicles containing 500 μM lipid dispersed in PBS. Values shown are the average from at least three samples, with generally no more than a $\pm$1 nm difference in individual samples relative to the average value. Unless otherwise noted, a similar degree of variability in λ_{max} was found in subsequent experiments. The width of the hydrophobic core of DOPC bilayers is about 30 Å. A two-carbon change in acyl chain length results in a corresponding change in bilayer width of about 3.5 Å (*23*).

to the membrane surface (*3*). However, this does not eliminate the possibility that oligomerization contributes to the red shift (see Discussion). In bilayers formed by the short-chain lipids DPoPC and DMoPC, previous studies suggest the red shift reflects the combination of helix oligomerization with a trivial effect arising from the intrinsically closer proximity of a Trp at the bilayer center to the bilayer surface in thin bilayers (*3*, *23*).

In thick bilayers, the red shift relative to the parent peptide [pL(L11)] was largest for the peptides containing H, K, or P substitutions [pL(H11), pL(K11), and pL(P11)]. In thin bilayers, Trp λ_{max} was not significantly dependent on sequence, with the possible exception of a slightly greater red shift for the peptide with a D substitution [pL(D11)] relative to the other peptides.

A larger range of λ_{max} values was observed for peptides that contain consecutive double amino acid substitutions for Leu at positions 10 and 11 (Figure 1B). The pL(G10G11) molecule behaved very similarly to the analogous peptide with a single substitution [pL(G11)]. However, the pL(S10S11), pL(P10P11), and pL(P10G11) peptides exhibited significant red shifts in thicker bilayers relative to analogous peptides with single substitutions (compare panels A and B of Figure 1). Even more strikingly, peptides containing two ionizable residues, pL(D10D11) and pL(K10K11), showed emission that was strongly red shifted at all bilayer widths. This highly red shifted fluorescence does not appear to be due to peptides dissolved in aqueous solution. The pL(D10D11) peptide, which showed the most red-shifted fluorescence and thus was the most likely to have dissociated from bilayers, was completely associated with both DOPC and DEuPC vesicles as judged by size exclusion chromatography on Sepharose 4B-CL (data not shown).

Determination of Peptide Location within Bilayers by Quenching of Trp Fluorescence: Effect of Sequence and Bilayer Width upon Peptide Location. To more fully characterize the effects of insertion-destabilizing substitutions, the degree to which the λ_{max} shifts observed above reflected changes in Trp depth, and thus peptide location within the bilayer, was determined. We concentrated on studies in lipid vesicles composed of DOPC, in which mismatch is minimal, and in thicker bilayers composed of DEuPC, in which there is considerable negative mismatch. To measure Trp depth, the dual quencher assay introduced in the accompanying paper was employed (*29*). In this method the level of quenching of Trp by bilayer-inserted molecules of 10-doxylnonadecane (10-DN) is compared to Trp quenching by acrylamide, which is predominantly located in aqueous solution. The more deeply a Trp residue is buried, the more strongly it is quenched by 10-DN and the more weakly it is quenched by acrylamide. The ratio of acrylamide quenching to 10-DN quenching (*Q*-ratio) is very sensitive to depth, with a low *Q*-ratio indicating a Trp location close to the bilayer center and a high ratio indicating a location near the bilayer surface (*29*). Trp depth can be specified more precisely by comparison to a standard curve obtained with Trp of known depth [see accompanying report (*29*)].

Quenching data for acrylamide and 10-DN are shown in Table 1. As expected, in general the results from the individual quenchers show an inverse correlation, with an increase in acrylamide quenching as 10-DN quenching decreases. Figure 2A shows *Q*-ratios calculated from these data for peptides incorporated in DOPC bilayers. For reference, *Q*-ratios for a Trp at the bilayer center [i.e., residue

Table 1: F_o/F Values (±SD) for Individual Quenchers

	DOPC		DEuPC	
peptide	acrylamide	10-DN	acrylamide	10-DN
pL(L11)	1.10 ± 0.04	3.78 ± 0.10	1.26 ± 0.04	2.13 ± 0.06
pL(F11)	1.09 ± 0.04	3.08 ± 0.18	1.34 ± 0.07	2.98 ± 0.21
pL(S11)	1.11 ± 0.01	3.21 ± 0.46	1.17 ± 0.03	2.52 ± 0.52
pL(A11)	1.12 ± 0.03	2.75 ± 0.08	1.23 ± 0.03	2.13 ± 0.07
pL(G11)	1.15 ± 0.02	2.96 ± 0.13	1.28 ± 0.05	2.31 ± 0.09
pL(P11)	1.11 ± 0.01	2.25 ± 0.11	1.32 ± 0.04	1.66 ± 0.05
pL(D11)	1.26 ± 0.01	3.04 ± 0.18	1.54 ± 0.08	2.53 ± 0.16
pL(K11)	1.31 ± 0.02	3.01 ± 0.23	1.65 ± 0.07	2.17 ± 0.09
pL(H11)	1.48 ± 0.05	3.07 ± 0.14	1.45 ± 0.03	2.34 ± 0.06
pL(S10S11)	1.09 ± 0.07	2.63 ± 0.03	1.20 ± 0.22	2.04 ± 0.16
pL(G10G11)	1.15 ± 0.05	3.25 ± 0.22	1.28 ± 0.01	2.72 ± 0.25
pL(P10P11)	1.26 ± 0.02	3.28 ± 0.57	1.58 ± 0.04	1.91 ± 0.02
pL(P10G11)	1.31 ± 0.01	3.30 ± 0.46	1.53 ± 0.03	2.25 ± 0.07
pL(K10K11)	1.51 ± 0.02	1.92 ± 0.02	1.97 ± 0.01	1.27 ± 0.17
pL(D10D11)	1.68 ± 0.05	1.47 ± 0.03	1.44 ± 0.06	1.35 ± 0.06

13 in the pL(L11) = pL(W13) molecule] and for a TM peptide with a Trp located near the polar/nonpolar interface of the bilayer [i.e., residue 4 in pL(W4)] are shown at the left.

Figure 2A shows that the quenching ratios for almost all of the peptides with a single amino acid substitution are low (<0.2), indicating that they, like the parental pL(L11) peptide, have a Trp very close to the bilayer center and predominantly adopt a TM orientation when incorporated into DOPC bilayers (see Discussion). The pL(H11) peptide has a slightly elevated *Q*-ratio (0.23) suggestive of a mixture of a TM population with a very small amount of a shallower population (see below). Low *Q*-ratios indicative a TM orientation are also observed for several of the peptides with double substitutions when they are incorporated into DOPC bilayers [pL(G10G11), pL(S10S11), pL(P10P11), and pL(P10G11)]. In contrast, pL(K10K11) and pL(D10D11) both show higher quenching ratios when incorporated into DOPC vesicles. The pL(D10D11) peptide *Q*-ratio (1.45) is consistent with a Trp location in the polar headgroup region near the polar/nonpolar interface (*29*). Since both the core Asp residues and flanking Lys residues should be surface seeking, this implies a conformation in which the entire peptide is located close to the bilayer surface (see Discussion). An alternate possibility is TM structure in which the peptide forms a water-filled pore into which acrylamide can pass. Such a structure seems very unlikely given the overall hydrophobicity of this peptide and the weak quenching of its Trp by 10-DN.[3] Furthermore, a pore-forming structure is inconsistent with experiments showing that Co^{2+} is unable to cross bilayers in the presence of the pL(D10D11) peptide (*29*).

The pL(K10K11) peptide shows a quenching intermediate between that for a deeply and shallowly inserted Trp (*Q*-ratio = 0.55). This implies either the formation of a single conformation in which the Trp is not positioned at the bilayer center or a mixture of a TM state with a state in which the peptide is located close to the bilayer surface. As described below and in the Discussion, the former explanation appears to be correct.

[3] It would be expected that the Asp residues would face the hypothetical pore, which would imply that Trp does not. Thus, Trp should show significant sensitivity to quenching by 10-DN in such a structure, but this is not detected.

The dual quencher assay was also applied to the peptides incorporated in DEuPC bilayers (Figure 3A). In the case of the parental pL(L11) peptide, the *Q*-ratio is similar to that observed in DOPC vesicles. There is also a low quenching ratio (<0.22) for the pL(S11), pL(F11), pL(A11), pL(G11), pL(G10G11), and pL(S10S11) peptides. In other cases, peptides exhibited larger *Q*-values, consistent with an intermediate Trp depth or mixture of a population with a deep Trp with a small amount [for pL(H11) and pL(D11), *Q*-ratio = 0.3−0.35] or considerable amount [for pL(P11), pL(K11), pL(P10G11), and pL(P10P11), *Q*-ratio = 0.42−0.64] of a population with shallowly locating Trp (see Discussion). Finally, peptides with consecutive ionizable residues [pL(D10D11) and pL(K10K11)] exhibited a shallow Trp depth (*Q*-ratio > 1.25), consistent with a shallow peptide location. It should be noted that it is not clear how much shallower the Trp of the pL(K10K11) peptide (*Q*-ratio = 3.7) is than that of the pL(D10D11) peptide (*Q*-ratio = 1.26).[4] How *Q*-ratio depends on depth near the membrane surface has not been determined (*29*).

Comparison of quenching ratios to λ_{max} values (i.e., comparison of panel A to panel B of Figure 2 and panel A to panel B of Figure 3) shows a strong correlation between high *Q*-ratio values and λ_{max} red shifts, although there are some small differences. Thus, it appears that Trp λ_{max} in DOPC and DEuPC vesicles primarily reflects Trp depth.[5] For this reason, it is noteworthy that mismatch effects on λ_{max} are further exaggerated when peptides are incorporated into vesicles containing the 2:1 (mol/mol) DNPC/DEuPC mixture,[2] which forms the widest bilayers studied. Assuming λ_{max} also reflects Trp depth in this lipid mixture, consecutive polar substitutions are much more destabilizing to TM orientation in DNPC/DEuPC than in DEuPC, such that pL(P10P11) is fully located at the bilayer surface, and even pL(S10S11) has a somewhat shallow Trp depth (Figure 1B). Overall, peptide behavior shows that negative mismatch can markedly exacerbate the effects of insertion-destabilizing substitutions.

Detection of Multiple Peptide Populations Using Quencher-Induced λ_{max} Shifts. As noted above, some peptides showed intermediate *Q*-ratios and λ_{max} values, which might reflect the presence of mixtures of TM and surface-located states. In a previous study a mixture of TM and non-TM orientations in thick bilayers was inferred from intermediate λ_{max} values accompanied by a slight broadening (by a few nanometers) of emission spectra (*3*). In the present study, quencher-induced λ_{max} shifts were measured in order to detect the presence of multiple peptide orientations more unambiguously. This method is described in detail in the accompanying report (*29*). To summarize, in samples in which there are

[4] It is possible that some pL(K10K11) in DEuPC is not vesicle bound. However, pL(K10K11) shows a similar λ_{max} and two-thirds as much quenching by 10-DN as pL(D10D11) in DEuPC vesicles. Since pL(D10D11) is fully vesicle bound, it is likely that most pL(K10K11) is also vesicle bound.

[5] It should be noted that the agreement between quenching and λ_{max} suggests that there are no artifacts due to specific binding of acrylamide or 10-DN to peptides. Nevertheless, there might be some peptide sequences that tightly bind one of these quenchers. It might be possible to identify cases of strong binding to peptide by the appearance of unusually strong quenching relative to the peptides examined in this report. In any case, it is recommended that λ_{max} data always be collected to confirm the results obtained by quenching.

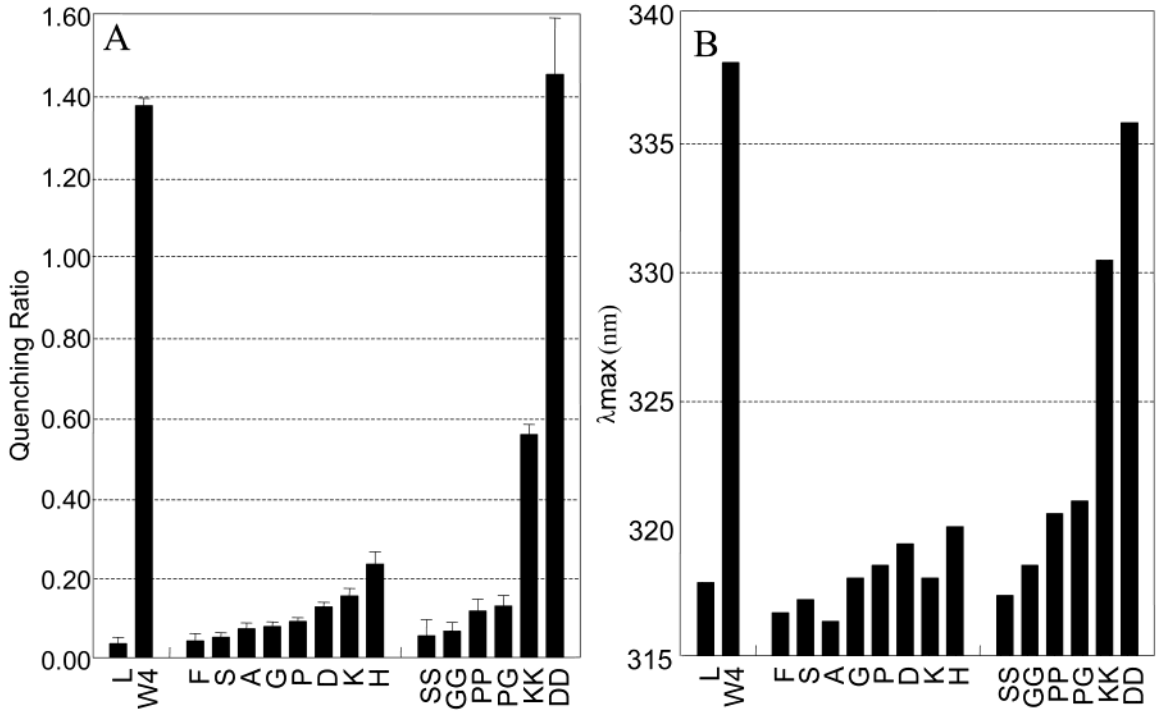

FIGURE 2: Depth and λ_{max} for peptides in DOPC vesicles. (A) Quenching ratios (*Q*-ratios) for peptides incorporated into DOPC. A high *Q*-ratio is equivalent to a shallow Trp location. Depth for the peptide pL(W4) shows the value for a Trp located at the polar/nonpolar boundary of the bilayer. Average and standard deviations for triplicates are shown. (B) λ_{max} for the peptides in panel A in the absence of quencher (data from Figure 1). Sample compositions are as in Figure 1, except when quenchers were present (see Experimental Procedures).

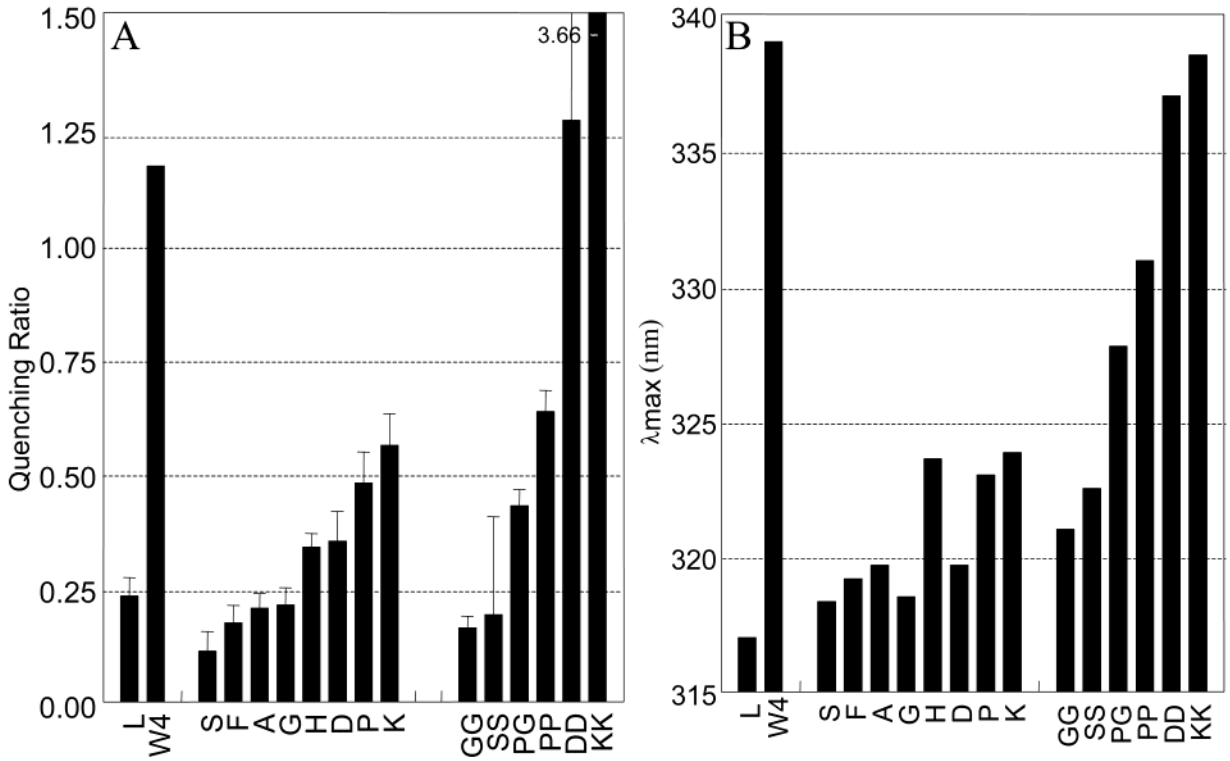

FIGURE 3: Depth and λ_{max} for peptides in DEuPC vesicles. (A) Quenching ratios for peptides incorporated into DEuPC. Average and standard deviations for triplicates are shown. Note that the off-scale *Q*-ratio for pL(K10K11) is 3.66. The off-scale standard deviations for the pL(D10D11) peptide and pL(K10K11) peptide were 0.28 and 2.3, respectively. (B) λ_{max} for the peptides in panel A in the absence of quencher (data from Figure 1). Sample compositions are as in Figure 1, except when quenchers were present (see Experimental Procedures).

two peptide populations with different depths (and thus different Trp λ_{max} values), acrylamide preferentially quenches the fluorescence of the shallower population and thus induces a blue shift, whereas 10-DN preferentially quenches the fluorescence of the deeper population and induces a red shift. For a homogeneous population large quencher-induced λ_{max} shifts should not be observed.

Table 2 shows the results of the λ_{max} shift assay. Only a small difference between λ_{max} in the presence of acrylamide and that in the presence of 10-DN (1−4 nm) was found for

Table 2: Shifts in Trp λ_{max} Induced by Acrylamide and 10-Doxylnonadecane for Peptides Incorporated in Lipid Vesicles

	λ_{max} (nm)			shift (nm)[a]	
peptide in	without quencher	+10-DN	+acryl-amide	+10-DN	+acryl-amide
DEuPC					
pL(L11)	321	322.5	318.5	1.5	−2.5
pL(S11)	318.5	321	317.5	2.5	−1
pL(P11)	323.5	330.5	320	7	−3.5
pL(P10P11)	333.5	341.5	325.5	8	−8
pL(D10D11)	337	337.5	335	0.5	−2
pL(K10K11)	338.5	339	336	0.5	−2.5
DOPC					
pL(P11)	320	320.5	319.5	0.5	0
pL(K10K11)	329	330	329.5	1	0.5

[a] A positive shift value indicates a quencher-induced red shift, and a negative shift value indicates a blue shift. The average of values from three samples is shown. Although average values are reported to the nearest 0.5 nm, the accuracy of individual values is about ±1 nm.

several peptides with deep Trp locations [pL(L11) and pL(S11) in DEuPC vesicles and pL(P11) in DOPC vesicles] and for peptides under conditions in which Trp location is shallow [pL(D10D11) and pL(K10K11) in DEuPC vesicles]. This confirms that these peptides form predominantly TM or shallow orientations, respectively. In contrast, two peptides giving an intermediate λ_{max} [pL(P11) and pL(P10P11) in DEuPC] showed a much larger difference (10−16 nm) between λ_{max} in the presence of acrylamide and that in the presence of 10-DN. This indicates that these peptides adopt a mixture of states with shallower and deeper Trp locations. A mixture of populations at different depths is also likely to exist for other peptides showing slightly red shifted λ_{max} values in DEuPC (see Discussion). In contrast, despite having an intermediate λ_{max}, pL(K10K11) in DOPC shows only small quencher-induced λ_{max} shifts. This indicates that this peptide predominantly forms a single conformation with an intermediate Trp depth. The nature of this conformation is considered in the Discussion.

The Location of Peptides Reflects an Equilibrium between Different States. Membrane-inserted peptides might adopt a specific conformation in response to a dynamic equilibrium or instead due to kinetic trapping occurring at the time of vesicle formation. To distinguish between these possibilities, decane addition experiments were performed. Exogenously added decane spontaneously inserts into the core of bilayers and can increase bilayer width significantly (*3*, *31*, *32*). For this reason, decane addition to bilayers can amplify the effects of hydrophobic mismatch, inducing a red shift in Trp λ_{max} for some peptides by increasing the fraction of surface conformation, but only if the peptides are not trapped in the TM state at the time of initial vesicle preparation.

Therefore, the influence of decane on peptides incorporated into DEuPC vesicles was examined (Figure 4A). Previous studies show that the maximum amounts of decane used in these experiments was sufficient to increase bilayer width to a degree equivalent to elongating the acyl chains by one to two carbon atoms (*3*).

Since pL(P10P11) forms a mixture of shallow and TM states in DEuPC, if not trapped in a particular mixture of conformations, it should be very sensitive to a decane-induced increase in bilayer width. Consistent with this prediction, a significant decane-induced red shift consistent

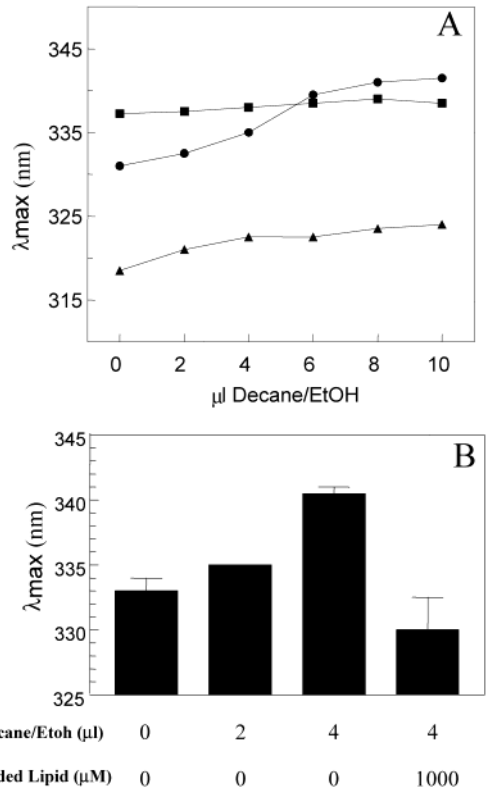

FIGURE 4: Effect of addition or dilution of decane upon λ_{max} of peptides in DEuPC vesicles. (A) Decane addition experiment for bilayer incorporated: pL(L11) (triangles), pL(P10P11) (circles), and pL(D10D11) (squares). Values of λ_{max} after sequential aliquots of 1:9 v/v decane/ethanol were added are shown. Samples contained 2 μM peptide incorporated into vesicles containing 500 μM DEuPC dispersed in PBS. (B) Decane dilution experiment for DEuPC-incorporated pL(P10P11). In a preincubation step 2 μL and then an additional 2 μL of 1:9 v/v decane/ethanol were added to 1 μM peptide incorporated into 125 μM DEuPC vesicles dispersed in PBS. The extent of the reversal of decane-induced shift upon addition of DEuPC vesicles lacking decane to a concentration of 1 mM lipid was then measured. Ethanol by itself had little effect on fluorescence emission for all of these samples (data not shown). Average and standard deviations for triplicates are shown.

with a decrease in Trp depth was observed in samples containing this peptide. In addition, a 38% increase in sensitivity to acrylamide quenching ($F_o/F - 1$) was observed in the presence of a high concentration of decane, also consistent with an increase in the fraction of this peptide near the bilayer surface (data not shown). The pL(L11) peptide showed a smaller red shift upon decane addition, consistent with the observation (see above) that it forms a much more stable TM state than that formed by pL(P10P11). No significant red shift was observed for the pL(D10D11) peptide upon addition of decane. This is expected as this peptide forms a shallow non-TM state in DEuPC and should not be affected by a further increase in bilayer width.

These experiments do not eliminate the possibility that once in a shallow orientation a peptide becomes trapped and cannot return to the TM state. To test this possibility, pL(P10P11) in DEuPC vesicles was preincubated with decane, and then an excess of vesicles lacking decane and peptide was added. The vesicles containing peptide should become thinner as the decane equilibrates into the decane-free bilayers.[6] As shown in Figure 4B, dilution of decane in this manner does significantly blue shift the Trp fluorescence of

pL(P10P11), indicating that shallowly oriented peptides can regain a TM conformation.

Circular Dichroism (CD) Shows That both TM and Surface-Located States Are Predominantly α-Helical. To monitor α-helix content, CD spectra were measured for the peptides incorporated in DOPC or DEuPC bilayers, and representative spectra are shown (Figure 5). For all of the sequences studied in this report the spectral shapes and intensities were those of predominantly α-helical polypeptides. To determine the percentage of residues in an α-helical conformation, the average of values determined from three different programs for calculating helix content was determined (see Experimental Procedures). In most cases the helix content calculated by the different programs was consistent within 5−10%. These analyses confirmed that the peptides formed highly helical structures (average 85 ± 8%).[7] However, pL(P10P11) peptide in DOPC and pL(P11), pL(P10P11), and pL(K10K11) peptides in DEuPC formed slightly less helix (roughly 70%). The decrease for the Pro-containing peptides is not surprising because Pro is a helix-breaking residue. There was no significant overall difference between peptide helix content in DOPC vesicles (86.5%) and DEuPC vesicles (84%).

DISCUSSION

Conformations Formed by Bilayer-Inserted Peptides. In this report Trp depth was used to determine the location of hydrophobic peptides in bilayers. It was found that insertion-destabilizing residues and lipid structure can combine to affect the conformation of hydrophobic poly(Leu) helices. However, before the effects of sequence and lipid properties can be considered in detail, the conformations formed by the inserted peptides and how they can be distinguished must be considered.

The formation of TM structures by hydrophobic Lys-flanked poly(Leu) peptides is well documented (*3*, *23*). For these peptides it has been shown that when the Trp at the center of the hydrophobic sequence locates at the bilayer center, it is indicative of a fully TM state (*3*, *23*). When the TM conformation is disrupted by introduction of insertion-destabilizing residues, a movement of the Trp away from the center of the bilayer is observed. One possibility for the resulting conformation is that the entire peptide moves close to the bilayer surface, such that both the charged helix-flanking Lys residues and the internal insertion-disrupting residue(s) are close to the bilayer surface (Figure 6). An alternative is that a truncated TM helix forms in which the insertion-destabilizing residue forms (or is near) one boundary of the TM segment (Figure 6) (*21*). In both of these conformations the Trp is closer to the bilayer surface than it is in the normal TM state. However, the Trp can be more shallowly located when the entire peptide moves close to the membrane surface than in a truncated helix (Figure 6).

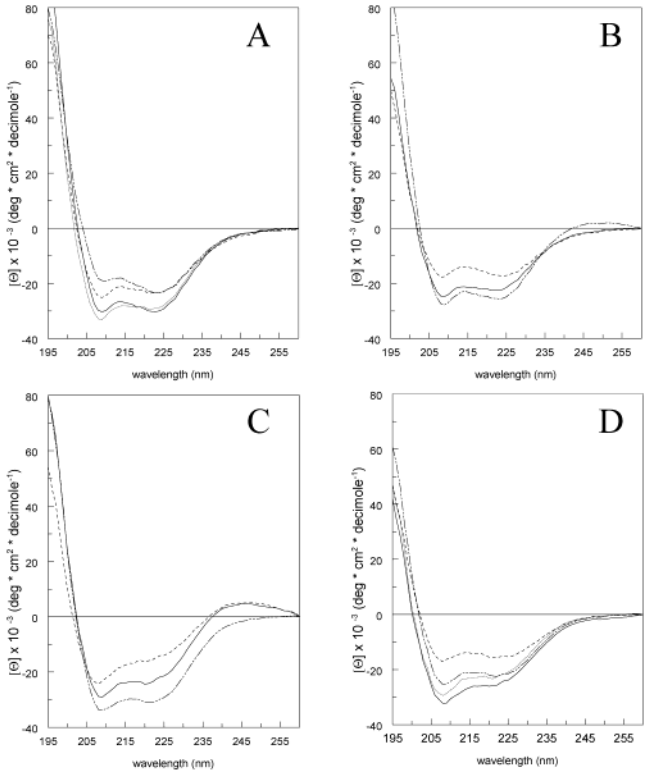

FIGURE 5: CD spectra of peptides incorporated in DOPC or DEuPC bilayers. Helix content was calculated as described in Experimental Procedures. (A) Peptides containing a single substitution incorporated in DOPC vesicles: pL(L11), 94% helix (solid line); pL(D11), 87% helix (dashes); pL(P11), 87% helix (dashes and double dots); and pL(A11), 79% helix (dots). The spectra for pL(K11), 94% helix, pL(H11), 94% helix, and pL(S11), 96% helix, were very similar to the spectrum for pL(L11). The spectra for pL(F11), 89% helix, and pL(G11), 93% helix, were very similar to the spectrum of pL(P11). (B) Peptides containing two substitutions in DOPC bilayers: pL(S10S11) (solid line), 81% helix; pL(P10G11) (dashes and double dots), 92% helix; and pL(P10P11) (dashes), 73% helix. The spectra for pL(G10G11), 83% helix, pL(K10K11), 82% helix, and pL(D10D11), 78% helix, were all very similar to the spectrum of pL(S10S11). (C) Peptides containing a single substitution in DEuPC bilayers: pL(L11), 90% helix (solid line); pL(F11), 96% helix (dashes and double dots); and pL(P11), 68% helix (dashes). The spectra for pL(G11), 90% helix, pL(A11), 86% helix, pL(D11), 87% helix, pL(K11), 92% helix, pL(H11), 88% helix, and pL(S11), 91% helix, were all very similar to the spectrum of pL(L11). (D) Peptides containing two substitutions in DEuPC bilayers: pL(S10S11) (solid line), 79% helix; pL(P10P11) (dashes), 67% helix; pL(P10G11) (dashes and double dots), 86% helix; and pL(D10D11) (dots), 80% helix. The spectrum for pL(G10G11), 84% helix, was similar to the spectrum of pL(P10G11), and the spectrum of pL(K10K11), 72% helix, was similar to that of pL(D10D11). Lipid and buffer backgrounds have been subtracted from each spectrum. Sample compositions are as in Figure 1 except that buffer was diluted 10-fold with water.

Effect of Hydrophobic Mismatch on the Stability of Various Peptide Conformations. The relative energy of the normal TM state, truncated TM state, and surface conformations should be dependent upon both hydrophobic helix length and bilayer width. In previous studies we found that an 15-residue Leu-rich sequence could form a stable TM structure when inserted into DOPC bilayers while one composed of 11 residues could not. Thus, the minimum Leu-rich hydrophobic segment allowing stable TM insertion in DOPC must be in the range of 11−15 residues (*23*). Poly(Leu) peptides in

[6] An alternate interpretation of these experiments is that the excess vesicles lacking decane fused with the decane-containing vesicles. This possibility was eliminated by measuring fusion via changes in energy transfer between NBD- and rhodamine-labeled lipids (*2*). No decrease in energy transfer was observed when a vesicle population containing pL(P10P11), decane, and 1 mol % of both NBD-labeled and rhodamine-labeled phosphatidylethanolamine was mixed with a population of excess vesicles lacking peptide, decane, and fluorescently-labeled lipids at the same concentrations as given in Figure 4B.

[7] The small difference in secondary structure compared to that we have previously reported for pL(L11) probably reflects the different methods used for analysis (*3*).

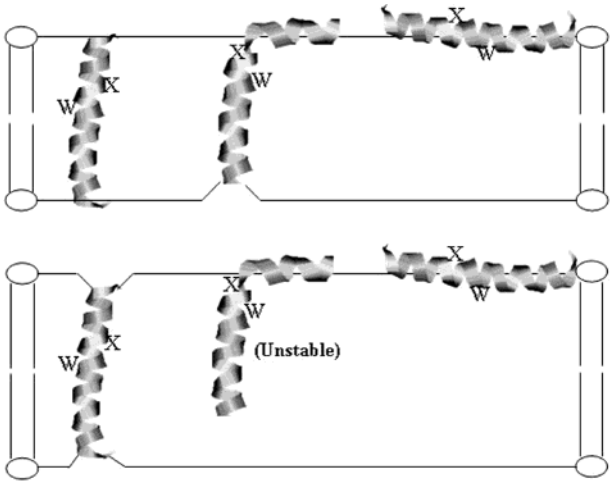

FIGURE 6: Potential conformations of poly(Leu) peptides in bilayers of various widths. Potential conformations of peptides shown are (left) normal transmembrane helix, (center) truncated transmembrane helix in which an insertion-destabilizing residue (X) is near the bilayer surface, and (right) near-surface peptide location. (Top) Intermediate width (e.g., DOPC) bilayer matching the length of the peptide hydrophobic core. (Bottom) Wide bilayer (e.g., DEuPC) with width exceeding the length of the peptide core (negative mismatch conditions). Bilayer distortions are shown adjacent to peptides for cases of negative mismatch in which bilayer shape adjustment may relieve some mismatch. Any oligomeric interactions are not illustrated. The Trp position is shown by W. Note that in the truncated transmembrane structure the segment that does not span the bilayer is shown as associated with the bilayer. This segment may be in equilibrium with a conformation in which it protrudes into aqueous solution.

which an insertion-disrupting residue at position 11 is located close to the bilayer surface might form an 11-residue-long truncated hydrophobic TM helix composed of residues 12−22. This would obviously be close to the borderline for stable TM insertion in DOPC bilayers. In contrast, those peptides that bury an insertion-destabilizing residue deep within the bilayer could form the normal TM state with a continuous 19-residue helix spanning the hydrophobic core of DOPC bilayers. In DEuPC bilayers [which form bilayers with a hydrophobic core about 7 Å wider than those formed by DOPC (*23*)], a TM helix formed by a 19-residue hydrophobic sequence would be somewhat destabilized by negative mismatch, whereas an 11-residue hydrophobic sequence would be so unstable in the TM state as to prevent its formation. In contrast to TM states, a conformation in which the entire peptide is aligned close to the bilayer surface would have an energy that was very largely independent of bilayer width and, thus, negative mismatch. Thus, when Trp depth and its dependence, or lack of dependence, upon bilayer width are combined, it becomes possible to identify the conformation formed by membrane-inserted poly(Leu) peptides (see below).

It should be noted that, in addition to altering peptide conformation, negative mismatch could be relieved by thinning of the lipid bilayer in the immediate vicinity of the peptide or by the formation of oligomers that stabilize the TM state by minimizing the surface area over which mismatch with lipid occurs (*4*, *15*, *33*). The data in this report show that these phenomena are often insufficient to allow maintenance of TM orientation. However, a small degree of oligomerization may occur under conditions of negative mismatch. We previously demonstrated that loosely associated oligomers can induce a red shift of Trp fluorescence that is dependent on peptide concentration within the bilayer (*23*). When the protein/lipid ratio for peptides with single substitutions was increased by 2.5-fold, a small red shift in Trp fluorescence was observed in some cases, suggesting a small degree of oligomerization (unpublished observations). We cannot rule out that a residual degree of oligomerization is even present at the more dilute peptide concentrations used in most of our experiments. However, the highly blue shifted Trp fluorescence found in the presence of single substitutions is inconsistent with a large extent of oligomer formation (*23*).

Sequence Dependence of Peptide Conformation and How It Is Affected by Mismatch: Effect of Ionizable (Asp or Lys) Residues in a Hydrophobic Sequence. The most dramatic examples in which conformations other than the normal TM state predominate are peptides with consecutive ionizable residues (AspAsp or LysLys) in their hydrophobic core. Instead of a normal TM helix, it appears that the pL(D10D11) peptide resides near the surface in both DOPC and DEuPC vesicles. These conclusions come from the observations that pL(D10D11) formed a state having very red shifted fluorescence and a very shallow Trp depth in both DOPC and DEuPC vesicles, and since a truncated TM helix would not be stable in DEuPC bilayers, the peptide must reside close to the membrane surface in DEuPC bilayers. Since its Trp depth and λ_{max} in DOPC and DEuPC are very similar, this peptide must also take on the surface conformation in DOPC bilayers.

The pL(K10K11) peptide in DEuPC also shows a very shallow Trp depth and highly red shifted λ_{max} values that are, moreover, very similar to that for the pL(D10D11) peptide, implying that it must also reside close to the membrane surface in DEuPC vesicles. In contrast, pL(K10K11) shows a lesser Trp red shift and intermediate Trp depth in DOPC vesicles. The demonstration that these intermediate values are not due to a mixture of populations with shallow and deep Trp depths (see Results) indicates this peptide predominantly forms a single conformation in DOPC that is different from that it forms in DEuPC. The depth of Trp in DOPC is inconsistent with formation of the normal TM conformation. One alternate possibility is that, in DOPC, pL(K10K11) lies along the bilayer surface but is somewhat more deeply buried than it is in DEuPC. However, this possibility must be rejected because we can identify no plausible physical mechanisms that would explain the existence of two distinct surface conformations with stabilities that are sensitive to bilayer width.

Instead, it is highly likely that the pL(K10K11) peptide forms a truncated TM structure in DOPC. This would explain its intermediate Trp depth and λ_{max}. In addition, the instability of a truncated helix in DEuPC vesicles would explain why the peptide converts to the surface conformation in DEuPC. This explanation would also account for the observation that the DD- and KK-containing peptides adopt different sturctures in DOPC. The relatively long alkyl chain of Lys can show "snorkeling" behavior (*20*, *34*, *35*) in which its amino group locates near the bilayer surface while the hydrocarbon part of the Lys chain remains deeper in the bilayer. As a result the amino groups of K10 and K11 could approach the bilayer surface without pulling the truncated hydrophobic helix formed by residues 12−22 very far toward the surface. However, for Asp carboxyl groups to locate at the membrane

surface, it would be necessary to pull one end of its truncated TM helix significantly closer to the surface (Figure 6, bottom). Thus, the movement induced by an Asp residue would expose more hydrophobic residues to a polar environment. Indeed, previous studies by Monne and von Heijne demonstrate that Asp residues in the core of hydrophobic peptides induce larger movements than Lys residues (*20*). In addition, the large movement necessary to move the Asp residues close to the bilayer surface would force Lys residues on the C-terminus of the peptide (residues 24 and 25) to become too deeply buried in the bilayer. Together, these differences would make formation of a truncated helix in DOPC vesicles much more unstable for pL(D10D11) than it is for pL(K10K11). Presumably, the truncated helix formed by pL(K10K11) is just long enough to stably span a DOPC bilayer.

This conclusion is supported by preliminary experiments with a peptide containing an Asp residue at position 9. Such a peptide should be able to form a truncated hydrophobic helix two residues longer than that formed by pL(D10D11) (i.e., composed of residues 10−22 instead of residues 12−22), and it shows behavior that mimics that of pL(K10K11) (unpublished observations).

In contrast to the behavior of consecutive ionizable residues, single ionizable residues (D, K, or H) are unable to prevent formation of the normal TM insertion in DOPC vesicles. However, in wider DEuPC bilayers, some decrease in average Trp depth was observed, especially for a peptide with a K substitution. Given the discussion above, this decrease in Trp depth presumably arises from a small population of peptides that have moved to the bilayer surface. Evidently the presence of single ionizable residues very close to the center of the hydrophobic sequence results in borderline stability for the TM form of poly(Leu) helices. It should be noted that, in a sequence less hydrophobic than that of poly(Leu) peptides, a single ionizable residue might have a much stronger influence on conformation.

It should also be noted that Vogt et al. (*36*) also found that the number of Lys residues in a hydrophobic sequence controlled TM stability. They found that poly(Leu) peptides with three and four Lys aligned along the bilayer surface, while two *non*consecutive Lys gave a broad distribution of orientations.

The most obvious explanation for why ionizable residues destabilize the normal TM conformation so strongly is that charged groups deeply buried in a bilayer would have extremely high energy. However, the behavior of peptides with single ionizable residues indicates that a TM structure in which deeply buried ionizable residues are uncharged can be more stable than one in which the peptides are at the surface and the ionizable residue is charged (*12*, *13*). Thus, it may be more correct to say that the energetic cost of Asp protonation or Lys deprotonation at neutral pH, added to the unfavorable energy for burial of these residues in their uncharged but polar form, inhibits their burial deep within bilayers.

Sequence Dependence of Peptide Conformation and How It Is Affected by Mismatch: Effect of Pro Residues in a Hydrophobic Sequence. The effects of Pro substitutions are less marked than that of ionizable residues. Consecutive Pro residues strongly destabilized the normal TM orientation in DEuPC bilayers but not in DOPC bilayers. Trp depth and the effect of quenchers upon Trp λ_{max} for the ProPro-containing peptide showed that it forms a mixture of structures in DEuPC bilayers. The very blue and red shifted λ_{max} values in the presence of acrylamide and 10-DN, respectively, are strongly suggestive of a mixture of the normal TM and surface states. This conclusion is strengthened by the fact that a truncated TM state should not be stable in DEuPC. In the even wider 2:1 DNPC/DEuPC bilayers (*3*) a more highly red shifted fluorescence characteristic of the surface state predominates. A single Pro substitution results in behavior similar to that in the presence of two Pro substitutions, although the peptide adopted a shallow location to a lesser extent.

Presumably, one reason that Pro is unfavorable in TM helices is that the missing amino proton leaves an unsatisfied H-bond acceptor, a carbonyl group, one helical turn away. This represents the minimal disruption of H-bonding by Pro. With two consecutive Pro there seems to be a more extensive decrease in helix formation (about 15%, which would involve 3−4 residues) and, thus, a presumably more extensive disruption of backbone H-bonding. For this reason, the observation that two consecutive Pro residues do not disrupt TM orientation in DOPC is somewhat surprising. One possible explanation is that pL(P10P11) forms a TM oligomer in which Pro faces away from lipid and can form interpeptide hydrogen bonds.

It should be noted that, in addition to an unfavorable energy for Pro insertion into bilayers, peptide behavior may be influenced by effects arising from the shortening of its effective hydrophobic length by a Pro-induced kink (*10*). This would increase the sensitivity of TM insertion to mismatch in wide bilayers. [A small bending effect has also been proposed for Ser residues and may influence the behavior of Ser-containing helices to a lesser degree (*37*).]

It should be noted that, in the presence of double insertion-destabilizing substitutions, we do not know the relative contributions of the substitutions at residue 10 and residue 11 to destabilization of the TM state. However, Trp depth data showing that the pL(P10G11) peptide in DEuPC vesicles shows a reduction in the level of formation of the TM state that is similar to that observed for pL(P11) in DEuPC vesicles suggest that both substitutions at positions 10 and 11 contribute significantly to the destabilization of the TM state.

Sequence Dependence of Peptide Conformation: Ser and Other Residues. A consecutive SS substitution had even less effect than two Pro residues. The pL(S10S11) peptide apparently retains a TM conformation in both DOPC and DEuPC. However, this peptide gives red-shifted fluorescence in 2:1 DNPC/DEuPC, indicating it may form a large amount of the surface state under conditions of extreme mismatch.

The other substitutions examined had at most small effects on the stability of TM insertion. This is not surprising as in most cases the substitutions involved also have little effect on hydrophobicity. Even a double substitution with the least hydrophobic residue of this group, Gly, was insufficiently hydrophilic to prevent TM insertion under conditions of extreme mismatch.

Correlation of Synthetic Peptide Behavior with Propensities for Membrane Insertion in Natural Proteins and Its Implications. It is noteworthy that the relative propensities of different residues to disrupt TM insertion found in this report correlate well with the inverse of their abundance in

(apparently) monomeric natural TM helices (*5*), decreasing in the order ionizable residues > Pro > Ser > hydrophobic residues. This inverse correlation indicates that studies of membrane-inserted synthetic peptides under conditions in which the stability of the TM state is varied are a valid strategy for developing a direct and detailed relationship between sequence and the stability of TM insertion. It should be a particularly useful system for analysis of positional effects and interactions between different polar and ionizable residues in hydrophobic helices.

Potential Biological Implications of the Effects of Mismatch and Its Sequence Dependence. The cumulative effects of mismatch and sequence are interesting because they show that lipid bilayer properties can have significant effects on hydrophobic helix conformation. Since different eukaryotic cellular membranes have different lipid compositions and may have different bilayer widths (*38*), it is conceivable that the structure and, thus, function of membrane proteins that move between different organelles are significantly altered by the changes in membrane properties that they encounter. Of course, it must be kept in mind that the peptides studied here are unusual in that they have very short hydrophilic flanking sequences. On the other hand, membrane proteins containing helical hairpins with a short linking sequence might well show TM insertion with a stability that is sensitive to bilayer structure in the same way as the peptides studied in the report. Furthermore, although a TM protein bounded by two large hydrophilic domains would be unlikely to respond to mismatch by losing its TM orientation, a hydrophobic TM segment with an appropriate sequence might convert between truncated TM helix and long TM helix states in response to local lipid environment (*3*).

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