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Folding of β-Sheets in Membranes: Specificity and Promiscuity in Peptide Model Systems

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The interactions that drive the folding of β-barrel membrane proteins have not been well studied because there have been few available model systems for membrane β-sheets. In this work, we expand on a recently described model system to explore the contributions of interstrand hydrogen bonds, side-chain/side-chain interactions and side-chain/membrane interactions to β-sheet formation in membranes. These experiments are based on the observation that the hydrophobic hexapeptide acetyl-Trp-Leu-Leu-Leu-Leu-Leu-OH (AcWLLLLL) folds, cooperatively and reversibly, into oligomeric, antiparallel β-sheets in phosphatidylcholine membranes. To systematically characterize the important interactions that drive β-sheet formation in membranes, we have used circular dichroism spectroscopy to determine the membrane secondary structure of each member of a complete host-guest family of related peptides of the form AcWLL-X-LL, where X is one of the natural amino acids. Peptides with hydrophobic X-residues of any size or character (X = Ala, Val, Ile, Leu, Cys, Met, Phe and Trp) form similar β-sheets in membranes, while peptides with any polar X-residue or Gly or Pro at the X-position are random-coils, even when bound to membranes at high concentrations. The observed membrane sheet preferences correlate poorly with intrinsic sheet propensity scales measured in soluble proteins, but they correlate well with several membrane hydrophobicity scales. These results support the idea that the predominant interactions of the side-chains in membrane-bound β-sheets are with the membrane lipids, and that backbone hydrogen bonding is the major driving force for the stabilization of β-sheets in membranes.

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Introduction

The basic forces that contribute to protein folding and structure have long been identified.[1,2] Solution of the long-standing ''protein folding problem'' will come from the correct parameterization of these interactions. For soluble proteins, a wealth of structural and thermodynamic data along with computational and model system studies has led to appreciable progress in this area. For membrane proteins, especially β-barrel membrane proteins, progress has been slower because the interactions are weighted very differently[3–7] and must be measured separately in the context of a membrane-like environment. While some gains have been made in understanding the driving forces for α-helical membrane proteins,[3,8–12] the fundamental principles of folding and structure in β-barrel membrane proteins are much less well established. For example, reliable algorithms for identifying membrane β-barrels in genome databases have not yet been developed and there have been few, if any, successful examples of *de novo* membrane β-barrel design.

One reason that our understanding of membrane β-barrel folding is incomplete is that there have been few useful model systems for establishing the fundamental thermodynamic and structural prin-

Abbreviations used: CD, circular dichroism spectroscopy; POPC, palmitoyloleoylphosphatidylcholine.

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ciples for folding. However, a peptide model, acetyl-Trp-Leu-Leu-Leu-Leu-Leu-OH (AcWLLLLL), which assembles reversibly into oligomeric, antiparallel β-sheets in membranes, has been described recently.[13] In this work, we expand the characterization of that unprecedented model system to include a complete family of host-guest analogs of the form AcWLL-X-LL, which we are using to systematically examine the important interactions that contribute to β-sheet folding in membranes. Specifically, we are exploring the relative importance of backbone hydrogen bonding and side-chain/membrane interactions in determining β-sheet structure. These are the first systematic studies of β-sheet formation in membranes, and thus contribute to solving problems in the genomic identification, design and engineering of β-barrel membrane proteins.

Results

Selection of the model system

The hexapeptide AcWLLLLL interacts with membranes through a two-step pathway that includes membrane binding of monomeric random-coil peptides and subsequent assembly of oligomeric β-sheets in the membrane.[13] The relative population of bound monomer and bound oligomer is determined by the lipid concentration, the peptide concentration, and the temperature, but under most experimental conditions AcWLLLLL is predominantly in the form of membrane-bound oligomeric, antiparallel β-sheets.[13] The identification of β-sheet as the predominant secondary structure type was made using circular dichroism (CD) and Fourier transform infra-red (FTIR) spectroscopy.[13] Modeling of the highly cooperative binding curves showed that the sheets are assembled in membranes through a nucleation and growth process and contain 10 to 20 peptides each.

In this work, we sought to systematically characterize membrane β-sheet formation using a complete host-guest peptide model system related to AcWLLLLL. We began by demonstrating the feasibility of using the family AcWLL-X-LL in which we vary the middle residue, X, of the pentaleucine segment. Using binding data for closely related pentapeptides,[7] we calculated the fractional membrane binding of all 20 peptides of the form AcWLL-X-LL. The fractional binding data, shown in Table 1, were calculated for a lipid concentration of 1 mM, which was used in all subsequent CD measurements. The details of the calculation are given in the legend to Table 1. At neutral pH, the terminal carboxyl group is charged and the predicted fractional binding of monomeric peptide ranges from 0.002 (X = aspartate) to 0.58 (X = tryptophan). The median fractional binding at pH 7 is 0.06. At pH 2.5, the carboxyl group is uncharged and the predicted fractional binding of peptide increases substantially, ranging from 0.52 (X = lysine) to 0.99 (X = tryptophan). The median fractional binding at pH 2.5 is 0.85. These calculated values are for the monomeric random-coil states in buffer and membranes. However, a peptide that assembles into β-sheets in membranes will bind much better than predicted in Table 1 because of the cooperativity of β-sheet formation. For example, at 1 mM lipid, AcWLLLLL is predicted to be 14 % bound to membranes as a monomeric random-coil but is actually almost completely bound because of cooperative β-sheet formation.[13] Using this family of peptides, we can thus examine peptide secondary structure at pH 7, where bound monomer concentration is low and β-sheet formation requires cooperative binding, and we can determine secondary structure at pH 2.5 where all the peptides bind well to membranes independent of whether they form β-sheets. Based on these binding data, we concluded that the host-guest family of the form AcWLL-X-LL is well suited for a systematic characterization of sheet formation in membranes.

The binding data in Table 1 for the AcWLL-X-LL hexapeptides were calculated from experimentally measured binding for very similar pentapeptides of the form AcWL-X-LL; therefore, we expected them to be highly accurate. Nonetheless, we verified the calculations by using fluorescence titration[14] to measure the binding of several monomeric peptides directly at pH 7 and at pH 2.5. In all cases, the measured binding was in good agreement with the calculated values shown in Table 1.

Spectroscopic signatures of peptide β-sheets

In Figure 1, we show the characteristic CD spectra of AcWLLLLL in buffer and in membranes at pH 7 and pH 2.5. The peptide CD spectrum in buffer has a minimum at 200 nm that indicates random-coil structure.[15–17] The CD spectra of the peptide in membranes have a minimum at 214-219 nm and maximum at 198-202 nm that are strongly indicative of β-sheet secondary structure.[15–17] In fact, the β-sheet bands of AcWLLLLL in membranes are nearly identical with those from native β-barrel membrane proteins.[15] Using FTIR spectroscopy, we confirmed that the secondary structure of AcWLLLLL in membranes is β-sheet and showed that the sheets are antiparallel.[13]

For AcWLLLLL in membranes at pH 7, there is also a strong minimum at 228 nm that arises from the B_b band absorbance of the indole side-chain of tryptophan.[18,19] This aromatic band is observed for AcWLLLLL and other oligomeric, membrane-binding peptides that contain tryptophan.[13,20–22] Because AcWLLLLL β-sheets are antiparallel, we hypothesized that the effect of the polar C terminus was a major contributor to the asymmetry in the Trp side-chain absorbance, which gives rise to the Trp CD band at 228 nm. This idea was tested by measuring the CD spectra of AcWLLLLL β-sheets in membranes at pH 3, pH 7 and pH 11 (Figure 1). The Trp peak at 228 nm is absent when

Table 1. Fractional binding of AcWLL-X-LL peptides to 1 mM POPC vesicles

X-residue in AcWLL-X-LL	At pH 7		At pH 2.5
	ΔG (kcal/mol) water to bilayer[a]	Fraction bound to 1 mM POPC[b]	Fraction bound to 1 mM POPC[c]
Ala	−4.6	**0.04**	**0.85**
Arg	−4.5	0.03	**0.59**
Asn	−4.4	0.03	**0.74**
Asp	−3.6	0.01	**0.87**
Cys	−5.1	**0.09**	0.90
Gln	−4.2	0.02	**0.68**
Glu	−2.8	0.002	**0.85**
Gly	−4.8	0.06	**0.79**
His	−4.6	0.04	**0.53**
Ile	−5.1	**0.09**	0.90
Leu	−5.4	**0.14**	0.93
Lys	−4.3	0.03	**0.52**
Met	−5.0	**0.08**	0.89
Phe	−5.9	**0.29**	0.98
Pro	−4.4	0.03	**0.73**
Ser	−4.7	0.05	**0.85**
Thr	−4.7	0.05	**0.79**
Trp	−6.7	**0.58**	**0.99**
Tyr	−5.8	0.23	**0.97**
Val	−4.7	**0.05**	0.84

[a] Mole-fraction free energy[14] of water-to-bilayer partitioning for AcWLL-X-LL hexapeptides calculated from the experimentally determined binding data for monomeric random-coil pentapeptides of the form AcWL-X-LL, listed by Wimley & White.[7] We previously demonstrated thermodynamic additivity in these peptides,[7,34] and we have shown that the addition of one leucine residue decreases ΔG for membrane binding by 0.56 kcal/mol.[7] Therefore, at pH 7, ΔG (predicted for AcWLL-X-LL) = ΔG (measured for AcWL-X-LL) − 0.56 kcal/mol. We confirmed the accuracy of this calculation by measuring the binding of several of these peptides directly at pH 7. See the text for details.

[b] Calculated fractional binding of peptides at pH 7 to 1 mM POPC vesicles, the concentration of vesicles used in all circular dichroism measurements in this study. Fractional binding was calculated from the mole-fraction partition coefficients by fraction bound = $K_x^* L/(W + K_x^* L)$ where K_x is the mole-fraction partition coefficient, L is the molar concentration of lipid (0.001 M) and W is the molar concentration of water (55.4 M at 25 °C) . Mole-fraction partition coefficients are calculated by $K_x = e^{-\Delta G/RT}$. Large bold numbers represent samples for which the CD spectra are shown in Figures 1-5.

[c] Calculated fractional binding of AcWLL-X-LL hexapeptides to 1 mM POPC vesicles at pH 2.5. Binding at pH 2.5 was calculated as described above, except that an extra 2.7 kcal/mol was subtracted from the free energy to account for the protonation of the C terminus.[7] These values have been confirmed experimentally for several of these peptides. Large bold numbers represent samples for which the CD spectra are shown in Figures 1-5.

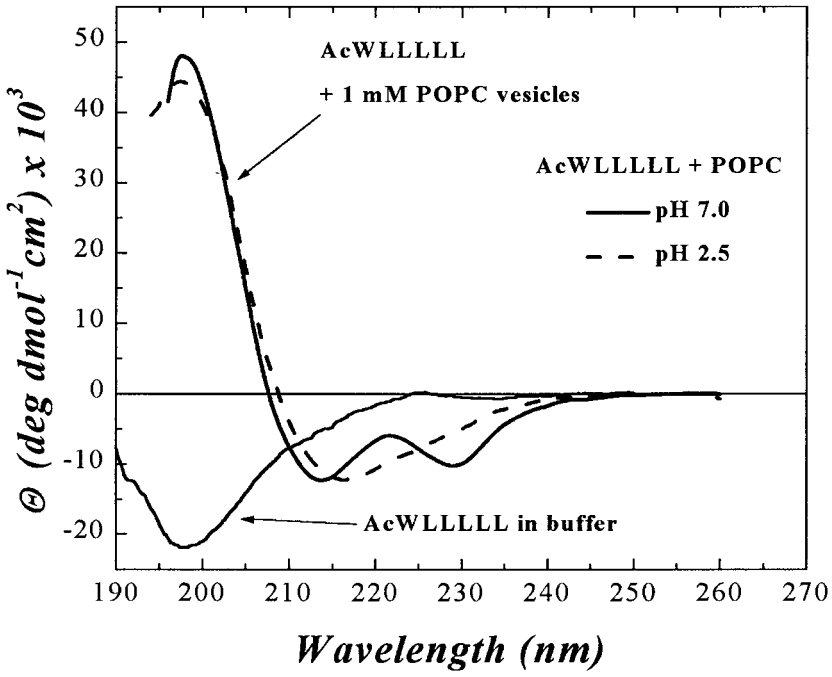

Figure 1. Spectroscopic signatures of peptide β-sheets in membranes. Circular dichroism spectra of AcWLLLLL at pH 7 in buffer, at pH 7 with 1 mM POPC vesicles and at pH 2.5 with 1 mM POPC vesicles. The buffer spectrum indicates random-coil while the membrane spectra indicate β-sheet secondary structure.

the C terminus is uncharged at pH 3 but is present at pH 7 and at pH 11 (not shown), where it is charged. We also tested the effect of *O*-methylation of the C terminus and found that AcWLLLLL-methyl ester readily formed β-sheets in membranes at all pH values, but did not have a 228 nm Trp peak at any pH value (not shown). These results support the idea that the β-sheets are antiparallel and that the Trp/carboxyl interactions in the antiparallel sheets are responsible for the sharp 228 nm peak.

The contributions of aromatic side-chains in all parts of the far UV CD spectra can be very large because there are aromatic transitions throughout the region between 200 and 230 nm[18] that overlap with the main β-sheet bands in those parts of the spectrum. Although these Trp side-chain contributions can affect the critical β-sheet bands at ~215 and ~200 nm,[23] the qualitative identification of β-sheet secondary structure from these bands is still possible in all the peptides we present in this work. In fact, the presence of the Trp band at 228 nm in the CD spectra of the analog peptides (Figure 2) is a sensitive indicator that the organization of these peptides in membranes is similar. Most importantly for this work, nearly all of the CD spectra can be identified unambiguously as either predominantly β-sheet or random-coil.

Membrane β-sheet formation at pH 7

For each member of the AcWLL-X-LL family, we measured CD spectra at pH 7 and pH 2.5 in solutions containing 1 mM large unilamellar vesicles[24] made from palmitoyloleoylphosphatidylcholine (POPC). Total peptide concentrations were 30-70 μM, and therefore the total system composition was 0.03-0.07 peptide molecule/lipid molecule. In all cases, the CD spectra are a weighted average of the membrane-bound and free peptide. In Figure 2, we show the CD spectra for all of the peptides that formed β-sheets in membranes at pH 7. Because all of these peptides are random-coils when dissolved in buffer, the fact that they show β-sheet structure by CD in the presence of membranes proves that these peptides are predominantly membrane-bound.

In the presence of membranes at pH 7, all of the peptides with simple hydrophobic X-residues (X = Val, Ile, Leu, Met, Phe and Cys), except for X = Ala, form β-sheets as shown by the CD spectra in Figures 1 and 2(a)-(e). AcWLL-A-LL is a random-coil under these conditions (Figure 2(f)). All of these CD spectra (Figure 2(a)-(e)) have the characteristic spectral features of AcWLLLLL β-sheets at pH 7, including the β-sheet bands at ~200 and ~215 nm, and the distinctive Trp peak at 228 nm. The peak positions are highly conserved for these peptides: 199.6(±1.3) nm, 215.2(±1.6) nm and 229(±0.6) nm. Several lines of evidence support the idea that the variability of the ellipticities in Figure 2 is not due to large differences in the peptide secondary structure. First, the ellipticities are independent of peptide concentration over the range 10-100 μM, indicating that the β-sheet peptides in this concentration range are not in equilibrium with a significant amount of random-coil peptide. Second, although the ellipticities vary for the β-sheet-forming peptides, the positions of the peaks are highly conserved. Third, the presence of the β-sheet peaks and Trp peak at 228 nm for the sheet-formers at pH 7 is strong evidence that the secondary structural arrangements are similar among these peptides, despite the large differences in the size and character of the X-residue side-chains. Fourth, like AcWLLLLL, the other β-sheet-forming peptides in Figure 2 are monomeric random-coils in buffer and are predicted to be only 5-29 % bound to the bilayers if they were interacting as monomeric random-coils (Table 1). Instead, the data in Figure 2 show that these hydrophobic sheet-forming hexapeptides bind very cooperatively to membranes and assemble into β-sheets in the same manner as AcWLLLLL.

AcWLL-A-LL

Because alanine also belongs to the class of simple hydrophobes, we further tested the propensity of AcWLL-A-LL to form β-sheets in membranes at pH 7 by making measurements at total peptide concentrations as high as ~200 μM. This concentration is close to the maximum aqueous solubility of the peptide. Even at these extreme concentrations, where the bound peptide to lipid ratios are as high as 0.008, we observed no indication of β-sheet formation for AcWLL-A-LL in membranes. At pH 2.5, on the other hand, the CD spectrum of AcWLL-A-LL at any concentration in the presence of membranes (Figure 2(f)) shows that it assembles into β-sheets. AcWLL-A-LL is the only peptide in the 20 member AcWLL-X-LL family that undergoes a clear coil to sheet transition between pH 7 and pH 2.5.

Near-UV CD analysis

We also performed a parallel set of near-UV CD measurements to confirm that the β-sheet-forming peptides are assembling into similar structures in membranes. Near-UV CD signals arise from the main absorption bands of the aromatic side-chains, in this case from the N-terminal tryptophan residue. The results, shown in Figure 3, strongly support the conclusions of the far-UV CD analysis. All of the β-sheet-forming peptides, X = Val, Leu, Ile, Cys, Met and Phe, have very similar near-UV CD spectra, with a broad minimum in the region around 285 nm, while peptides that are random-coils in membranes have only a small positive ellipticity or none at all in this region of the spectrum. For example, in Figure 3 we show the near-UV CD spectra for X = Trp, Tyr, Thr and Gly at pH 7 in the presence of membranes. These results strongly support our conclusion that all of the β-sheet-forming peptides have similar structure in

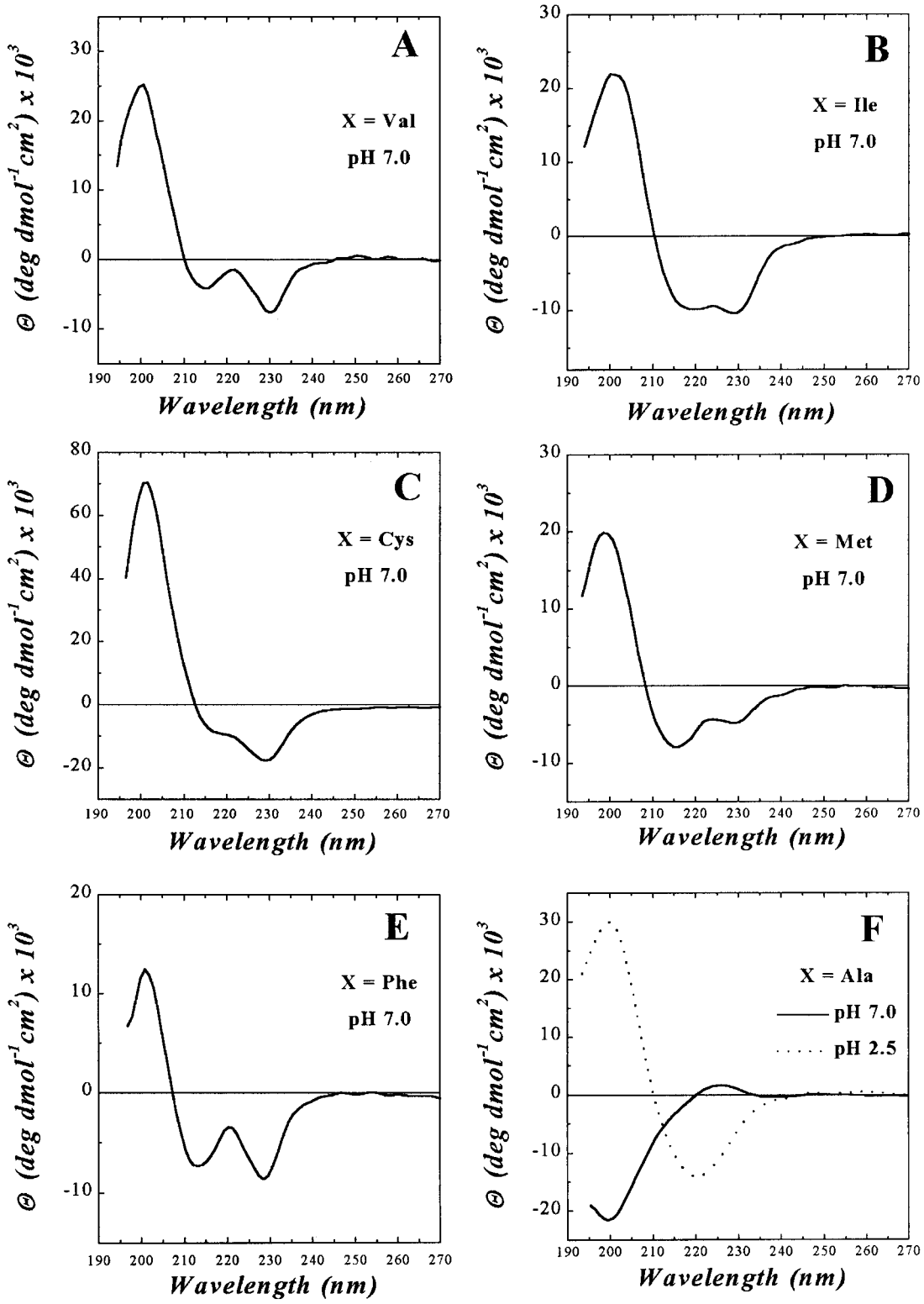

Figure 2. Far-UV CD spectra of β-sheet-forming AcWLL-X-LL peptides in the presence of POPC vesicles at pH 7. Experimental details of sample preparation and data analysis are given in the text. In each experiment, the peptide concentration is between 30 and 70 μM peptide, and the lipid concentration is 1 mM. All of the peptides shown in (a)-(e) have β-sheet secondary structure under these conditions. In (f) we show that AcWLL-A-LL is random-coil at pH 7 in the presence of membranes, but forms β-sheets in membranes at pH 2.5.

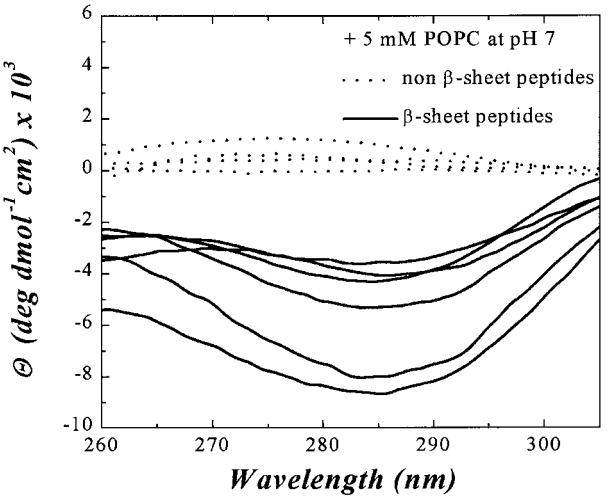

Figure 3. Near-UV circular dichroism spectra of AcWLL-X-LL peptides in the presence of POPC vesicles at pH 7. For these experiments, the concentration of POPC vesicles was 5 mM and the peptide concentration was 0.1-0.4 mM. Ellipticity is calculated per number of tryptophan residues. From top to bottom at 285 nm, non-sheet forming peptides are X = Trp, Tyr, Gly and Thr. The β-sheet-forming peptides, from top to bottom at 285 nm, are X = Ile, Leu, Val, Met, Phe and Cys. All of the β-sheet-formers have a broad negative CD band at ~285 nm while the random-coil peptides do not.

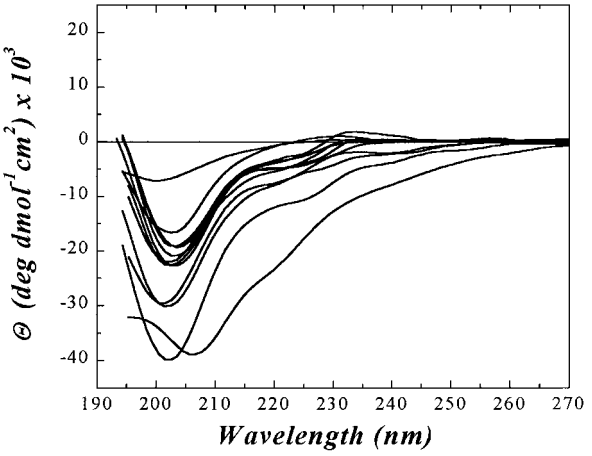

Figure 4. Circular dichroism spectra of non β-sheet-forming AcWLL-X-LL peptides at pH 2.5 in the presence of 1 mM POPC vesicles. Experimental details of sample preparation and data analysis are given in the text. In each experiment, the peptide concentration is between 30 and 70 μM peptide, and the lipid concentration is 1 mM. The predicted fraction of monomeric peptide bound is listed in Table 1. From top to bottom at 200 nm, the peptides are X = Pro, His, Glu, Arg, Gln, Lys, Asp, Asn, Gly, Thr, Tyr and Ser.

the β-sheet. At pH 2.5 neither the β-sheet-forming peptides nor the random-coil peptides had a substantial ellipticity in the near-UV region.

Peptide secondary structure at pH 2.5

For peptides that were random-coils at pH 7, the main contribution to the CD spectra is from monomeric, random-coil peptide in the aqueous phase (see Table 1), so we have not shown those spectra here. In order to probe the secondary structure of these peptides in the membrane we repeated the CD measurements at pH 2.5, where all peptides are mostly bound to the membranes (Table 1). Except for AcWLL-A-LL and AcWLL-W-LL, all the peptides that were random-coils at pH 7 were also random-coils when bound to membranes at pH 2.5, as shown by the CD spectra in Figure 4. These include X = Gly or Pro and all the amino acids that have polar groups in their side-chain: X= Tyr, Asp, Glu, Asn, Gln, Ser, Thr, His, Arg, Lys. At pH 7, where membrane binding is weak and most of the peptide molecules are in the aqueous phase, the random-coil CD minimum for these peptides occurs at 198.5(±1.5) nm and has an ellipticity of −20,100(±3100) deg dmol^{-1} cm^2. At pH 2.5, where membrane binding is strong and most of the peptides are in the membrane (Table 1), these same peptides have a very similar random-coil minimum at 202.5(±1.0) nm that has an average ellipticity of −22,600(±7900) deg dmol^{-1} cm^2. It is important to note that bound peptide/lipid ratios in the pH 2.5 random-coil peptides were in the range of 0.03 to 0.07. For comparison, AcWLLLLL assembles into β-sheets at pH 7 at bound peptide/lipid ratios as low as 0.001. Thus β-sheet formation in AcWLL-X-LL is inhibited strongly, even at high membrane concentration, when the X-residue side-chain is Gly or Pro, or when it contains any polar group.

The hydrophobic peptides that formed β-sheets in the presence of membranes at pH 7 (X= Val, Cys, Met, Leu, Ile and Phe) also had CD spectra at pH 2.5 indicative of β-sheet formation. These spectra are not shown, but they had an average minimum at 220.6(±1.7) nm with an intensity of 15,500(±1600) deg dmol^{-1} cm^2, and an average maximum at 200.8(±0.9) nm with an intensity of 50,300(±7400) deg dmol^{-1} cm^2. Near-UV CD spectra of β-sheet-forming peptides at low pH had little detectable ellipticity.

AcWLL-W-LL

The peptide AcWLL-W-LL is unique, in that its CD spectrum at pH 2.5, shown in Figure 5, cannot be ascribed unambiguously to either β-sheet or random-coil. The spectrum has strong minima at both 203 nm and 219 nm. There is no linear combination of sheet and coil spectra that will reproduce the observed spectrum. To show that this is not due to inherent conformational effects or aromatic contributions to the CD spectra in the monomeric peptide, we measured the CD spectrum of AcWLL-W-LL in buffer. In solution, AcWLL-W-LL

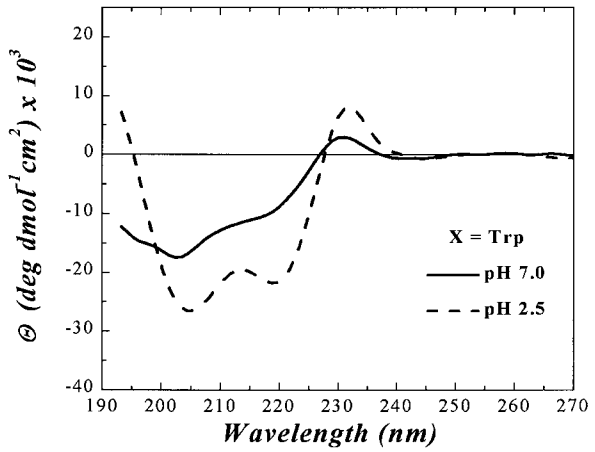

Figure 5. Circular dichroism spectra for AcWLL-W-LL in the presence of membranes at pH 7 and at pH 2.5. Experimental details of sample preparation and data analysis are given in the text. In each experiment, the peptide concentration is ~50 μM and the lipid concentration is 1 mM. The predicted fraction of monomeric peptide bound (Table 1) is 0.58 at pH 7 and 0.99 at pH 2.5.

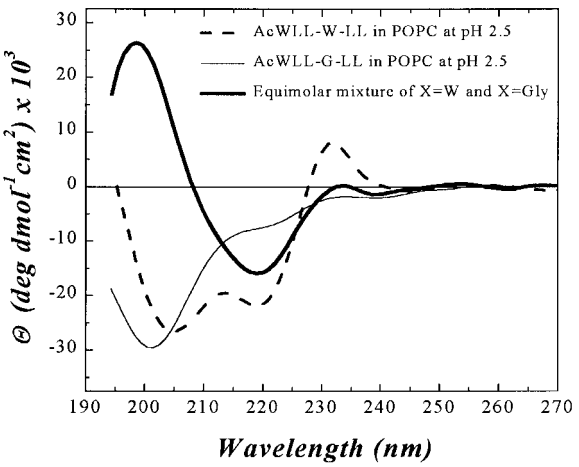

Figure 6. Circular dichroism spectra of AcWLL-G-LL, AcWLL-W-LL and a 1:1 mixture of both. These spectra were taken in the presence of 1 mM POPC vesicles at pH 2.5. In pure form, AcWLL-G-LL is a random-coil while AcWLL-W-LL has a CD spectrum that cannot be assigned unambiguously to sheet, to coil or to a linear combination of the two. The mixture is unambiguously β-sheet.

has a normal random-coil spectrum with only a broad minimum at 200 nm; therefore, AcWLL-W-LL must be forming secondary structure in membranes at pH 2.5. We cannot assign the secondary structure of AcWLL-W-LL to a specific type unambiguously; however, we note that the CD spectrum is very similar to that of indolicidin,[22] a 13 residue membrane-permeabilizing antibiotic peptide that has five tryptophan residues. Indolicidin was recently shown by NMR to form a β-turn conformation in membrane interfaces,[25] suggesting that AcWLL-W-LL in membranes at pH 2.5 may be forming a similar structure. It is possible that the backbone CD spectrum of AcWLL-W-LL is being obscured by the contributions of its two aromatic side-chains. To address the potential for AcWLL-W-LL to form β-sheets in membranes, we measured the CD spectra of binary mixtures of AcWLL-W-LL with AcWLL-G-LL. We chose AcWLL-G-LL for this β-sheet rescue experiment because, although it is not polar, it also does not form β-sheets in pure form, presumably because of its conformational flexibility. We also chose AcWLL-G-LL because the absence of the X-residue side-chain may minimize the aromatic X-residue contribution to the CD spectrum. The results are shown in Figure 6. At pH 2.5, the binary mixture of AcWLL-G-LL and AcWLL-W-LL has a spectrum that is indistinguishable from the β-sheet spectrum of AcWLL-A-LL at low pH (Figure 2(f)). This is observed for AcWLL-W-LL/AcWLL-G-LL ratios ranging from 1:1 to as high as 3:1. Thus, sheet formation in AcWLL-W-LL can be rescued by AcWLL-G-LL readily at low pH. We thus classify tryptophan as a sheet-forming amino acid in this system, although only a marginal one, like alanine, because it does not form sheets at pH 7.

Binary mixtures of AcWLL-G-LL with AcWLL-W-LL or AcWLL-Y-LL at pH 7 gave no indication of β-sheet structure. Nor did binary mixtures of AcWLL-Y-LL and AcWLL-G-LL at pH 2.5 as long as the total peptide concentrations were below about 80 μM. These experiments confirm our characterization of tryptophan as a marginal sheet-forming amino acid and tyrosine as a non-sheet forming amino acid in this model system.

The effect of glycine

The effect of glycine in these peptide β-sheets is worthy of closer examination, because glycine is one of the most abundant amino acids in the membrane-spanning β-strands of naturally occurring β-barrel membrane proteins (W.C.W., unpublished results). We hypothesized that the conformational flexibility of glycine in these small peptides inhibited β-sheet formation. In order to examine the magnitude of this effect, we synthesized AcWL-G-LLL, in which the glycine residue is at the third position and is expected to have a smaller conformational effect than in AcWLL-G-LL, where it occupies the central position. The results are shown in Figure 7. Both peptides are random-coils in the presence of membranes at pH 7, but at pH 2.5 AcWL-G-LLL forms β-sheets, while AcWLL-G-LL does not. This observation supports the idea that the conformational flexibility of glycine prevents β-sheet formation in AcWLL-G-LL.

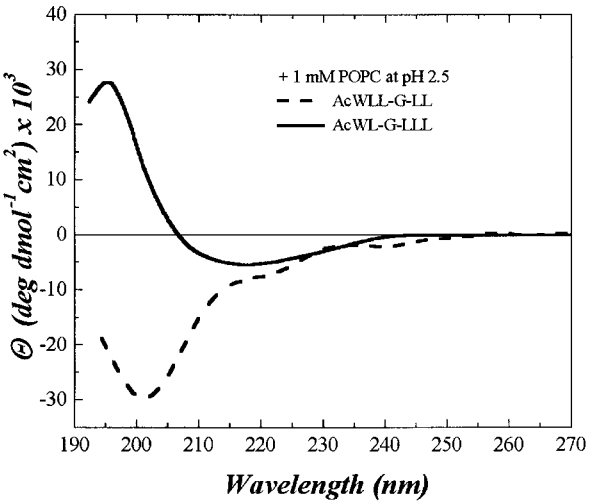

Figure 7. Circular dichroism spectra of ~50 μM AcWLL-G-LL and AcWL-G-LLL at pH 7 and pH 2.5 in the presence of 1 mM POPC vesicles. Both peptides are nearly fully bound to the vesicles at the low pH value, AcWL-G-LLL forms β-sheets and AcWLL-G-LL does not. See the text for details.

Fluorescence spectroscopy

To explore the environment of these peptides in membranes, we measured tryptophan fluorescence emission spectra for all the random-coil and β-sheet peptides in buffer and in membranes at pH 2.5. The average wavelength of emission maximum for the N-terminal Trp residues was 348(±1) nm in buffer, 331(±1) nm for the β-sheet formers in membranes and 334(±1) nm for the random-coil peptides in membranes. The 14-20 nm decrease in the Stokes shift upon membrane binding indicates that the Trp residues of all membrane-bound peptides are in an environment of reduced polarity, independent of secondary structure. These values of emission maxima are very similar to those observed for peptides that are known to bind in the region of the lipid carbonyl groups in the lipid bilayer interface.[26,27] However, because of the differences in secondary structure and oligomerization state between the β-sheet and random-coil groups, the fluorescence emission data alone are not sufficient to draw any more specific conclusions about the location of the terminal Trp residues within the membrane interface. Importantly, the fluorescence data unequivocally demonstrate that the N-terminal Trp residues are not buried deep within the hydrocarbon core of the membrane, where the emission maximum would be at around 320 nm.

Discussion

Information on the fundamental interactions that drive the folding of soluble proteins has come from studies with model systems,[28] and from equilibrium unfolding studies of proteins and their site-specific mutants.[29] Unfortunately, equilibrium unfolding methods such as calorimetry and spectroscopy are generally not applicable to membrane proteins,[30] and we must rely almost exclusively on model systems to measure the fundamental driving forces. While some model systems for α-helices in membranes have been developed,[3] there are few, if any, models for β-sheets in membranes, except for the system that we utilize in this work. This exception is based on the observation that a hydrophobic hexapeptide of the form AcWLLLLL reversibly and cooperatively forms oligomeric, antiparallel β-sheets in membranes.[13] Here, we have used a complete host-guest peptide family of AcWLLLLL analogs to systematically address the relative roles of interstrand hydrogen bonding, side-chain/side-chain interactions, and side-chain/membrane interactions in the folding of β-sheets in membranes.

We envisioned several testable hypotheses to explain membrane β-sheet formation in AcWLLLLL. One hypothesis is that sheet formation is driven by specific surface-surface interactions between AcWLLLLL molecules or between small AcWLLLLL sheets. This type of interaction is known to contribute to the dimerization of soluble leucine zipper α-helices[31] and the dimerization of membrane-spanning α-helices.[32] If surface/surface interactions are important, then these membrane sheets will be stacked and will have buried side-chains. The stability of such sheets would presumably be sensitive to the identity of the residues comprising the interacting surfaces.[33]

Alternately, if interstrand backbone hydrogen bonding in the membrane drives sheet formation, then we expect these peptide β-sheets to be single, unstacked sheets in the membrane, with the nonpolar side-chains interacting mainly with the lipids. In support of this model, previous measurements have shown that hydrogen bonding in membranes can, in principle, account for the observed structure propensity of peptides such as AcWLLLLL in membranes.[7,9,34,35] If true, this property would be in sharp contrast to the important role that specific side-chain/side-chain and side-chain/backbone interactions play in β-sheet formation in soluble proteins.[36–38] Furthermore, it would strongly suggest that the design and engineering of β-barrel membrane proteins and the identification of β-barrel membrane proteins in genome databases must be based on sequence pattern recognition rather than on specific details of amino acid sequence or intrinsic β-sheet propensity. For example, naturally occurring β-barrel pores have a dyad repeat sequence of alternating lipid-exposed hydrophobic residues and pore-lining hydrophilic residues.

Membrane β-sheet formation

The most important results of these experiments are that the AcWLL-X-LL peptides form similar β-sheets in membranes when the X-residue is any hydrophobic amino acid (X = Ala, Val, Ile, Leu, Cys, Met, Phe and Trp). Thus, these structurally similar peptide β-sheets can readily accommodate hydrophobic X-residues of any size or character in the X-position (see the molecular graphics image in Figure 8). This observation argues strongly against the possibility that these β-sheets are stabilized by specific surface/surface interactions between peptides or between small β-sheets, because such interactions would presumably be very sensitive to the size and character of the X-residue.

Importantly, these peptides are completely prevented from forming β-sheets in membranes when the X-residue contains any polar or charged group, or when it is glycine or proline. We can rationalize the effect of glycine in terms of its conformational flexibility and proline by its structure-breaking conformational properties and reduced H-bonding capacity. But how do polar groups on the X-residue side-chains prevent membrane β-sheet formation in AcWLL-X-LL? We eliminated membrane binding as the explanation for the inhibition of β-sheet formation by the polar X-residues by making the CD measurements at pH 2.5, where all 20 peptides bind well to membranes (see Table 1). Secondary structure was determined at concentrations of 30-70 μM peptide and 1 mM lipid or 0.03 to 0.07 bound peptide/lipid. This is about 50-fold higher than the concentration at which AcWLLLLL forms β-sheets at neutral pH, 0.001 bound peptide/lipid.[13] In addition, we expect that the reduction of unfavorable interaction involving the carboxy termini, when they become uncharged at low pH, will also increase the propensity for sheet formation. Yet, despite the high peptide concentration in the membrane and decreased unfavorable interactions at low pH, no peptide with a polar X-residue forms any detectable β-sheet in membranes. Based on the CD spectra for AcWLLLLL shown in Figure 1, we estimate that we can detect a β-sheet content of as little as 25 %.

How large an energetic cost is needed to prevent membrane β-sheet formation? We have successfully modeled the formation of β-sheets from AcWLLLLL[13] using a nucleation and growth model adapted by Terzi *et al.*[39] from Cantor & Schimmel:[40]

$$C_T = C_m\left[1 - \sigma + \frac{\sigma}{(1 - sC_m)^2}\right]$$

where C_T is the total membrane mole-fraction concentration of peptide and C_m is the mole-fraction concentration of bound monomeric peptide. The model contains a nucleation parameter, σ, and a growth parameter, s. Both parameters are expressed as mole-fraction equilibrium constants. For AcWLLLLL partitioning into POPC at pH 7, we measured values of $\sigma = 0.24$ (+0.84 kcal/mol at 298 K) and $s = 563$ (−3.75 kcal/mol at 298 K) (1 cal = 4.184 J). In Figure 9, we show the magnitude of the effects caused by increasing either the growth parameter free energy, or both free energies by 1 or 2 kcal/mol. Based on this simplified model we conclude that a $\Delta\Delta G$ of at least 2 kcal/mol for a Leu to X substitution is required to have the observed effect of preventing β-sheet formation in AcWLL-X-LL.

Sheet propensity scales from soluble proteins

How do the β-sheet preferences in the context of AcWLL-X-LL in membranes compare with sheet

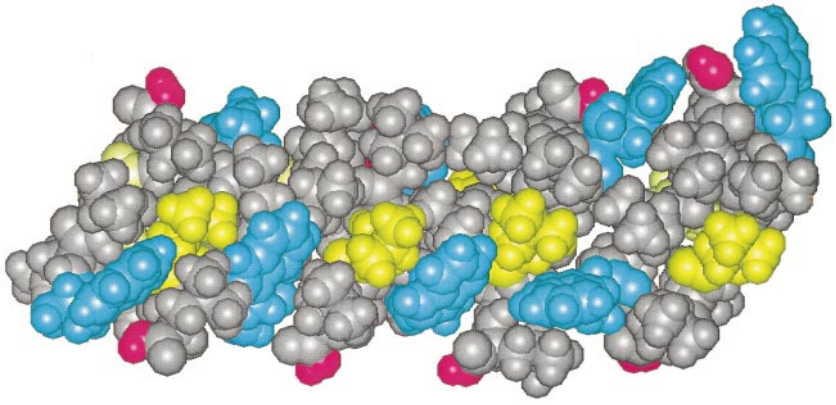

Figure 8. Molecular model of a possible octameric antiparallel β-sheet composed of AcWLLLLL. This model conforms roughly to what we know about these peptides, and is meant simply to show the dimensions of the sheet and the relative location of the termini and of the X-residue. The terminal carboxyl group is red, the N-terminal tryptophan residue is blue and the X-residue leucine is yellow. The construction of this simple molecular model is described elsewhere[13]. Note how the X-residues dominate the central portion of the sheet. Because these sheets are antiparallel, the other face is the same. The length of each peptide strand is ~20 Å from the Trp nitrogen atom to the terminal carboxyl group.

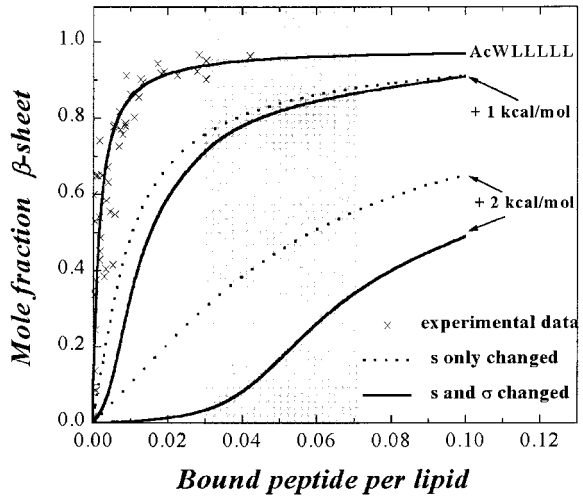

Figure 9. Modeling of the cooperative binding of β-sheet-forming peptides using a nucleation and growth model as described in the text. The uppermost curve was obtained by fitting the experimentally determined partitioning of AcWLLLLL into POPC vesicles as described.[13] The experimental data, shown here for reference, are published.[13] The other curves were calculated by adding 1 kcal/mol or 2 kcal/mol to either the growth parameter free energy, or to both the growth and nucleation parameter free energies. For reference, the concentration of bound peptide in our experiments at pH 2.5 was 0.03-0.07 peptide/lipid, as shown by the gray box.

propensities found for soluble proteins? Comparisons are shown graphically in Figure 10, where we have plotted the values of β-sheet propensity from three widely noted scales.[41–43] Amino acids that promote membrane β-sheet formation in AcWLL-X-LL are shaded, and those that prevent membrane β-sheet formation in AcWLL-X-LL are open. Overall, the correlation of intrinsic β-sheet propensity in soluble proteins with sheet preference in AcWLL-X-LL in membranes is poor. Also, the free energy range in the two experimentally determined free energy scales is small compared to the minimum cost of 2 kcal/mol that we estimated in Figure 9 was needed to prevent β-sheet formation in AcWLL-X-LL. In the two free energy scales, all of the amino acids, except proline, fall within ±2 kcal/mol of leucine and most of them fall within ±1 kcal/mol. We conclude from this comparison that the intrinsic β-sheet propensities measured in soluble proteins cannot be used to rationalize the β-sheet preferences that we observed for AcWLL-X-LL in membranes. Especially noteworthy are the positions of tyrosine and threonine; they are the most potent β-sheet-formers in soluble proteins, and yet they strongly inhibit β-sheet formation in these model peptides in membranes. Clearly, the fundamental interactions that drive sheet formation in membranes are very different from those that drive sheet formation in soluble proteins.

Cross-strand interactions in soluble proteins

Mutational studies by Smith & Regan[37] showed that cross-strand hydrophobic interactions, including aromatic ring-stacking, hydrogen-bonding, and electrostatic interactions can stabilize β-sheets in soluble proteins if the interacting residues are paired across adjacent solvent-exposed sites. Such interactions can contribute as much as 1 kcal/mol of stabilization.[37] These specific, non-local interactions probably contribute to the context-dependence of β-sheet propensity in soluble proteins.[36,37] Could such cross-strand interactions account for our observation that only the hydrophobic residues drive sheet formation in membranes? Although some contribution cannot be ruled out, it is unlikely to completely explain our observations, for several reasons. First, these peptide β-sheets are forming in membranes where the hydrophobic effect is absent or reduced, and where polar and electrostatic effects are enhanced.[7,35] For these reasons, we would expect a smaller contribution from cross-strand hydrophobic interactions and a greater contribution from sheet-promoting, hydrogen-bonding residues like tyrosine and threonine. Instead, we observed the opposite effect. Second, tyrosine and threonine, for example, in various combinations show strong pairwise contributions to stability in solvent-exposed positions on soluble β-sheets,[37] whereas they prevent sheet formation completely in our peptides. Third, the extra cross-strand interaction energies observed by Smith & Regan were less than 1 kcal/mol relative to the intrinsic propensities of the mutated residues. In the context of our model system, this contribution would be insufficient to account for the observed differences in β-sheet formation in our peptides (see Figure 9). Nonetheless, we cannot entirely rule out cross-strand interactions as a contributor to membrane β-sheet formation. Additional experimental studies are currently underway to specifically address this issue in more detail for peptide β-sheets in membranes.

Membrane hydrophobicity scales

Can the observed membrane β-sheet preference be accounted for in the interactions of the side-chains with the membrane itself? In Figure 11 we have plotted the relative water to membrane partitioning free energy for four hydrophobicity scales that have been applied to membrane proteins. These scales differ in the specific part of the bilayer environment that they mimic or represent. For example, the Goldman, Engelman & Steitz (GES)[44] and Radzicka & Wolfenden (RW)[45] scales assume very non-polar environments that may mimic the hydrocarbon core of the membrane, where the cost of partitioning charged side-chains and polar groups is large. On the opposite extreme, the Wimley & White bilayer scale (WW bilayer) is based on the partitioning of small peptides into the interfacial region of a bilayer,[7] a less hydrophobic

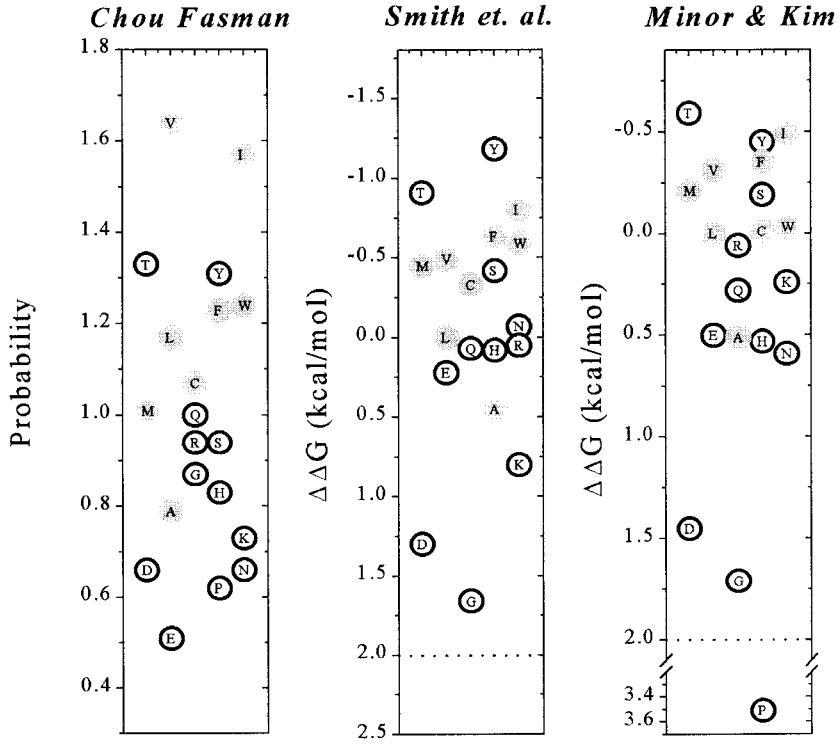

Figure 10. β-Sheet propensity scales for the amino acids in soluble proteins. In each panel, one propensity scale is shown. The residues that support β-sheet formation in AcWLL-X-LL in membranes are shaded and those that prevent β-sheet formation are open. The scales are: Chou & Fasman,[41] the classical probability scale derived from the abundance of each amino acid in β-sheets in proteins of known structure; Minor & Kim,[43] an experimental scale derived from mutational studies of a domain of staphylococcal protein G; and Smith *et. al.*,[42] also derived from mutational studies of a domain of protein G. The free energies are given as $\Delta\Delta G$, because they are the changes in ΔG induced by mutations at a solvent-exposed site on a β-sheet. The values have been shifted so that $\Delta\Delta G$ for leucine = 0. The dotted lines in the rightmost two panels signify the value of 2 kcal/mol lower free energy than leucine. This is the minimum free energy difference that we estimated in Figure 9 was required to prevent sheet formation in AcWLL-X-LL. The points have been spread randomly along the horizontal axis for clarity.

environment that is more permissive of polar moieties. The WCW octanol scale is for peptide partitioning into bulk octanol,[34] intermediate in polarity between the non-polar and interfacial phases of the other scales.

Among these scales, the two that are based on the non-polar hydrocarbon core environment (GES and RW) show excellent correlation with our observed β-sheet preferences. The eight sheet-forming amino acids in the context of AcWLL-X-LL are the eight most hydrophobic residues and the range of free energies in these two scales is consistent with the minimum free energy change of ~2 kcal/mol that we estimated was necessary to prevent β-sheet formation in AcWLL-X-LL. Note that the marginal β-sheet-formers in our model system, Ala and Trp, are also near the bottom of the set of sheet-formers in the hydrophobicity scales. The two scales based on more polar environments (WW bilayer, WW octanol) have a poorer correlation with the β-sheet preferences observed for AcWLL-X-LL, and the free energy range of these hydrophobicity scales is too small to account for our observations. For example, in both of these scales, the less hydrophobic sheet-forming residues, such as alanine, valine and cysteine, are interspersed among amino acids such as tyrosine, threonine and serine that are nearly as hydrophobic, but which completely prevent β-sheet formation in AcWLL-X-LL.

Structure, design and engineering

In this work, we found that peptide β-sheet formation in membranes is promiscuous, in the sense that peptides with any hydrophobic residue of any size form very similar β-sheets in membranes. We attribute this behavior to the fact that β-sheet formation in membranes is driven mainly by enhanced hydrogen bonding in the membrane environment. Furthermore, we conclude that these peptide β-sheets are assembling into single unstacked sheets in the membrane in which the side-chains interact mainly with the bilayer lipids. On the other hand, there is also a physicochemical specificity in the absolute requirement that these peptide β-sheets contain no polar groups of any nature in the X-position. We thus concluded that the X-residue side-chains of these single, isolated peptide β-sheets are interacting with the non-polar hydrocarbon core of the lipid bilayer.[46]

In soluble molecules, such single isolated sheets have been observed or designed only rarely,[47–49] and they require very specific cross-strand interactions between the amino acid side-chains. The situation may be very different in membranes, where the promiscuous, or non-specific, hydrogen bonding contribution can be dominant and is entirely able to support the formation of isolated secondary structure in membranes. If membrane β-sheet formation in membranes is stabilized mainly by promiscuous hydrogen bonding inter-

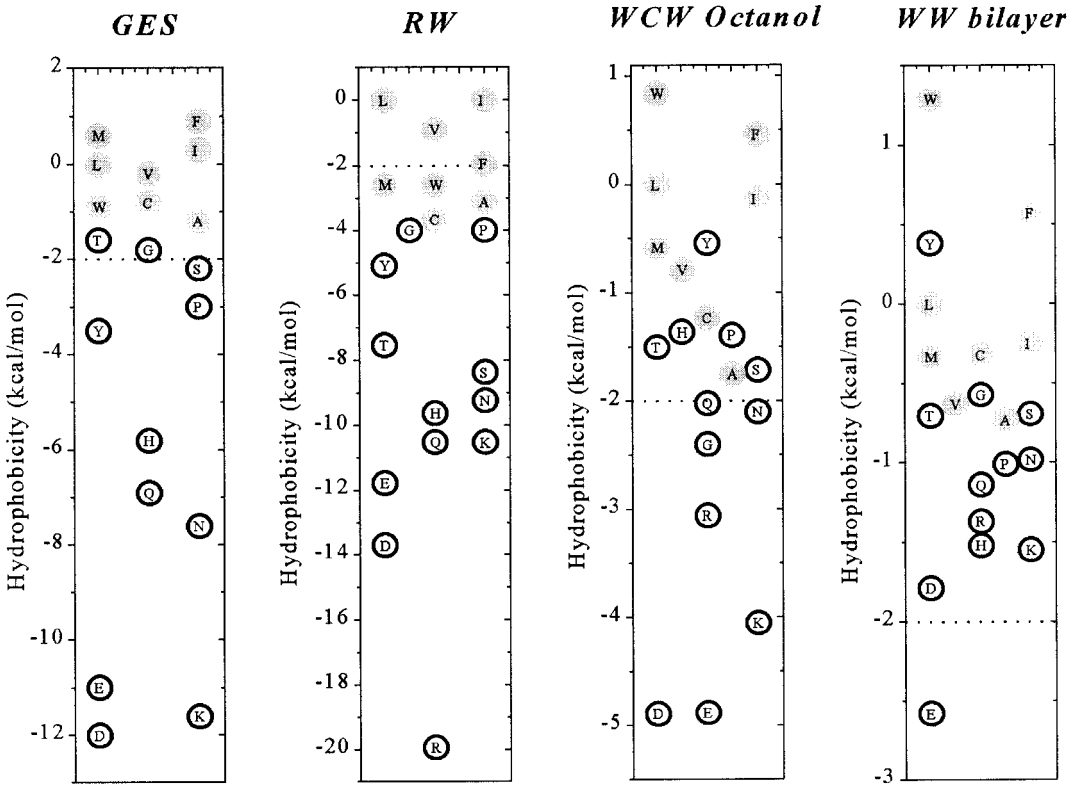

Figure 11. Hydrophobicity scales for the amino acids in membranes. In each panel, one hydrophobicity scale is shown with the values shifted so that the value of leucine is zero. The residues that support β-sheet-formation in AcWLL-X-LL in membranes are shaded and those that prevent β-sheet-formation are open. The scales are: GES, Goldman, Engelman & Steitz,[44] a contrived scale based on side-chain partitioning into cyclohexane and other data; RW, a scale based on the partitioning of amino acid side-chain analogs into cyclohexane;[45] WCW octanol, Wimley, Creamer & White,[34] an experimental scale based on the partitioning of small peptides into bulk octanol; WW bilayer, an experimental scale based on the partitioning of small peptides into bilayer interfaces.[7] The free energies have been shifted so that ΔG for leucine = 0. The dotted line in each panel signifies the value of 2 kcal/mol lower free energy than leucine. This is the minimum free energy difference that we estimated in Figure 9 was required to prevent sheet formation in AcWLL-X-LL. The points have been spread randomly along the horizontal axis for clarity.

actions, as these peptide studies have strongly suggested, then the specificity in the sequence-structure relationship in β-barrel membrane proteins may be derived, at least in part, from the interactions of a putative β-sheet's lipid-exposed surfaces with the lipid bilayer environment. A very different approach may thus be necessary for the design and engineering of β-barrel membrane proteins; one based on patterns of hydrophobic and hydrophilic amino acids rather than on very specific atomic-level design principles.

Materials and Methods

Materials

The buffer used in all experiments was 50 mM potassium phosphate (pH 7.0) or potassium phosphate buffer titrated to pH 2.0 with phosphoric acid. pH 2.5 buffer was prepared *in situ* by mixing one part of pH 7 buffer with two parts of pH 2 buffer. Peptides were synthesized manually using FMOC chemistry and were purified with reverse-phase HPLC. Details are given elsewhere.[7,13,26,34] Large unilamellar vesicles were made by extrusion under nitrogen pressure through two stacked, 0.1 μm pore Nucleopore polycarbonate filters. Vesicles prepared by this method are uniform unilamellar vesicles of 0.1 μm diameter.[24,50] The lipid we used was palmitoyloleoyl phosphatidylcholine (POPC) purchased from Avanti Polar Lipids.

Circular dichroism (CD) spectroscopy

CD spectroscopy was done on an OLIS RSM CD spectrometer. All measurements were made in a rectangular quartz cuvette with a pathlength of 1 mm. Vesicles were present at 1 mM, and we were able to measure CD with no absorbance flattening[51] down to a wavelength of ~195 nm. Near-UV CD spectra in the range of 260-320 nm were made in 1 mm cuvettes using lipid concentrations of 5 mM and peptide concentrations in the range of 0.1 to 0.4 mm. CD samples were prepared in several different ways in order to check for proper peptide incorporation and equilibration. All gave identical results. These methods have been described in detail elsewhere.[13] We made pH 2.5 samples by first preparing a 3× concentrated solution at pH 7 and then decreasing the pH to 2.5 by the addition of a twofold excess of pH 2.0 PO_4 buffer. Samples were then equilibrated for

30-60 minutes at 50 °C before CD measurements, which were made at 22 °C.

In the absence of lipid vesicles, large visible particles of peptide precipitate formed in five to ten minutes after acidification. Most of the peptide in solution could be removed from solution by mild centrifugation. Interestingly, these samples had no detectable CD signal, even before centrifugation, presumably because the peptide precipitate particles were too large to allow the passage of any UV light. Low-pH samples made in the presence of lipid vesicles contained no visible precipitate, and none of the peptide could be removed from the samples by centrifugation. Therefore, the peptides are fully incorporated into the vesicles at pH 2.5.

Samples made with the cysteine-containing peptide AcWLL-C-LL were prepared fresh from dry peptide and their CD spectra were measured within 30 minutes of preparation. Immediately afterward, the samples were acidified to prevent disulfide bond formation. We used HPLC to confirm that no disulfide-linked peptide dimers formed during the measurements. In all cases, peptide concentration in the CD samples were measured by HPLC after the CD measurement.[13,52]

Fluorescence spectroscopy

Tryptophan fluorescence emission spectra were measured in an SLM-Aminco 8100 spectrometer. All samples contained 0.3 mM POPC vesicles and 2-10 μM peptide. Excitation was at 270 nm and light-scattering from the vesicles was reduced with cross-polarization using Glann-Thompson polarizers. All the important principles of these measurements can be found elsewhere.[53]

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