Experimentally determined hydrophobicity scale for proteins at membrane interfaces

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The partitioning of membrane-active oligopeptides into membrane interfaces promotes the formation of secondary structure. A quantitative description of the coupling of structure formation to partitioning, which may provide a basis for understanding membrane protein folding and insertion, requires an appropriate free energy scale for partitioning. A complete interfacial hydrophobicity scale that includes the contribution of the peptide bond was therefore determined from the partitioning of two series of small model peptides into the interfaces of neutral (zwitterionic) phospholipid membranes. Aromatic residues are found to be especially favoured at the interface while charged residues, and the peptide bond, are disfavoured about equally. Reduction of the high cost of partitioning the peptide bond through hydrogen bonding may be important in the promotion of structure formation in the membrane interface.

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Many membrane-active peptides, including small toxins[1], antimicrobial peptides[2] and signal sequences[3], partition into membrane interfaces where they gain secondary structure. The processes by which these peptides partition and fold may provide important clues to the folding and stability of membrane proteins. The insertion and folding of membrane proteins can occur spontaneously[4] or result from a complex translocation process that uses metabolic energy[5]. The polypeptide chain must, in either case, make a transition into the membrane during which it folds into its native structure. In one model of this process, the native structure is attained through sequential stages of interfacial binding, secondary structure formation, and insertion of secondary-structure units into the membrane bilayer[6]. Thus, the partitioning and folding of small oligopeptides in membrane interfaces are closely related to the folding and assembly of membrane proteins.

A fundamental requirement for a quantitative description of protein partitioning and folding in membrane interfaces is a suitable hydrophobicity scale. In order to understand the coupling between partitioning and folding, the scale should describe the partitioning of peptides that lack regular structure so that the energetic consequences of folding can be evaluated relative to an unfolded state. We present such a scale for the twenty natural amino acids and the peptide bond derived from measurements of the partitioning of two families of small peptides into large unilamellar vesicle (LUV) membranes formed from palmitoyloleoylphosphatidylcholine (POPC). One family consisted of the compete set of host-guest pentapeptides acetyl-WL-X-LL-OH (AcWL-X-LL) with X being any of the twenty natural amino acids and the other a homologous series of peptides, $AcWL_m$ with m=1–6. We recently reported on the partitioning of these two families between octanol and water[7] and on the determination of the energetics of salt bridges that form in the pentapeptides between the X=Arg or Lys and the C terminus[8]. Those measurements and the present ones allow a direct experimental comparison of octanol and membrane partitioning. Because octanol is commonly used for measurements of bulk-phase hydrophobicities[9–11], this is a useful comparison.

Membrane and octanol hydrophobicities are found to be strongly correlated, but the interfacial values are generally about one-half those observed for octanol. The whole-residue interfacial values (that is, side chain plus peptide backbone) fall into three distinct classes: all of the charged residues are highly unfavourable, the aromatics are highly favourable, and the remaining residues make smaller contributions. The cost of transferring a peptide bond into the membrane interface is, remarkably, as costly as transferring a charged side chain. This high cost is likely to figure prominently in the formation of secondary structure in membrane interfaces.

Peptides and their disposition in membranes

The principal design criteria for the host-guest peptides were that partition coefficients be measurable for all twenty of the natural amino acids. An appropriate balance between non-polar and aqueous-phase solubility was achieved by the relatively high non-polar composition of the peptides on the one hand and their small size and charged C termini on the other. Additional design criteria were that the peptides be soluble in water as monomers, lack well defined secondary structure in aqueous and bilayer phases, and that the

guest (X) residue be in a covalent environment characteristic of unfolded proteins. Except for $AcWL_6$ that was not used in computations, the AcWL-X-LL and $AcWL_m$ peptides satisfied all of these criteria. The Trp_1 residue was included because of its usefulness as a spectroscopic probe.

The expected consequence of the lack of secondary structure was that the peptides would partition exclusively into the membrane interfaces. The interfaces, defined by the distribution of the water associated with the headgroups, are each about 15 Å thick and consist of a complex and thermally disordered mixture of water and headgroups and methylenes from the edges of the hydrocarbon core[12]. Several lines of evidence indicate that the pentapeptides partition into these interfaces without deep penetration into the hydrocarbon core. First, ^2H NMR measurements of the acyl chain order parameter profiles of POPC membranes with partitioned AcWL-W-LL indicate an interfacial location for the peptide (Klaus Gawrisch, personal communication). Second, neutron diffraction measurements show directly that the closely related peptide A-W-A-*O*-*tert* butyl is located in the bilayer interface with the Trp residue located mostly outside the hydrocarbon core[6]. Third, NMR measurements show that the disposition of A-F-A-*O*-*t*-Bu is similar to that of the Trp tripeptide[13]. Fourth, the possibility of completely partitioning a pentapeptide with 'open' hydrogen bonds into a hydrocarbon phase is low. Roseman[14] has estimated that the cost of partitioning a non-hydrogen bonded peptide bond into a hydrocarbon phase is 5–6 kcal mol^{-1} which is significantly larger than the free energy reduction[7,9] of ~3 kcal mol^{-1} associated with the partitioning of the most hydrophobic amino acid side chains. Given these numbers, our pentapeptides should be virtually insoluble in an alkyl phase. To confirm this expectation, we attempted to measure the partitioning of our most hydrophobic peptides between buffer (low pH) and n-octane but could not detect the peptides in the octane phase. We conclude that our peptides must be located in the interfacial region of LUV membranes rather than fully immersed in the hydrocarbon core. Furthermore, based on the instrumental sensitivity of the HPLC instrument used to determine peptide concentrations in octane, we estimate the free energy cost of partitioning non-hydrogen bonded backbone NH/CO pairs into an alkyl phase to be 4 kcal mol^{-1} or greater, consistent with Roseman's estimate[14].

Whole residue interfacial hydrophobicities

Mole-fraction partition coefficients between buffer and membrane interface were determined using equilibrium dialysis and reverse-phase HPLC[15]. Most measurements were made at pH 8 but some were made at pH 2 in order to determine the effect of protonating the C terminus. The resulting free energies of transfer ΔG from membrane to water for the AcWL-X-LL and $AcWL_m$ (m=1–6) peptides are listed in Tables 1 and 2 respectively. Fig. 1 shows that ΔG for bilayer partitioning of $AcWL_m$ increases linearly with peptide length m+1, just as it does for octanol partitioning[7], but with a least-squares slope $\Delta\Delta G_{Leu}$ of 0.56±0.05 kcal mol^{-1} per

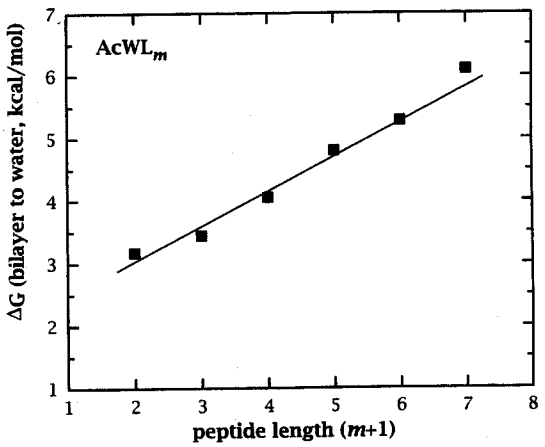

Fig. 1 Free energies of transfer of $AcWL_m$ (m=1–6) peptides from bilayer interface to water as a function of peptide length, m+1. Partition coefficients were measured using equilibrium dialysis and HPLC (see Table 1 footnotes) and free energies calculated using mole-fraction units[15]. Experimental uncertainties are approximately the size of the symbols. The partitioning of $AcWL_6$ is potentially complicated by aggregation in the water phase[7] so the linear regression analysis includes only m=1–5. The slope of the least-squares line is 0.56±0.05 kcal mol^{-1} per Leu.

residue which is about one-half the octanol value. The whole-residue free energies of partitioning $\Delta G_X^{residue}$, shown in Table 1 for all of the amino acids, are derived from the pentapeptide free energies ΔG_{WLXLL} (Table 1) at pH 8 using:

$$\Delta G_X^{residue} = \Delta\Delta G_{Leu} + (\Delta G_{WLXLL}^{pH8} - \Delta G_{WLLLL}^{pH8}) \qquad (1a)$$

for uncharged and negatively charged guest residues and:

$$\Delta G_X^{residue} = \Delta\Delta G_{Leu} + (\Delta G_{WLXLL}^{pH2} - [\Delta G_{WLLLL}^{pH8} - \Delta G_{COO}]) \qquad (1b)$$

with pH 2 ΔG_{WLXLL} values for positively charged residues. Eq. 1*b* can also be used for Asp and Glu in order to evaluate the energetic consequences of their protonation. Although a value of ΔG_{WLLLL} determined experimentally at pH 2 is preferable to the calculated value used in Eq. 1*b*, the limited aqueous solubility of the peptide precluded accurate measurements.

ΔG_{COO} in Eq. 1*b* is the free energy change associated with protonating the C terminus and was found to be 2.68±0.10 (SEM) kcal mol^{-1} using the differences in free energies of the Gly, Ala, Ser, and Thr peptides determined at pH 8 and pH 2. This is considerably smaller than the value of 4.78 kcal mol^{-1} observed for partitioning into octanol[7]. Because the partition coefficient of a protonated peptide is so much larger than that of a deprotonated peptide, one must be certain that only the deprotonated form is present at pH 8. The value of ΔG_{COO} and the pK_a of the C terminus in the aqueous phase, known[8] to be 3.7, indicate that the apparent carboxyl pK_a in the interface is 5.7, consistent with a negligible population (~0.5%) of the neutral form at pH 8. Furthermore, the partition coefficients were independent of pH between pH 7 and pH 9, indi-

Table 1 Free energies of transfer of AcWL-X-LL peptides[1] from bilayer interface to water

X-residue	pH	$^{2}\Delta G_{WLXLL}$ (kcal mol^{-1})	$^{3}\Delta G_{X}^{residue}$ (kcal mol^{-1})
Ala	8	4.08 ± 0.03	−0.17 ± 0.06
	2	6.94 ± 0.02	
Arg	8	3.91 ± 0.02	
	2	6.12 ± 0.02	−0.81 ± 0.11[4]
Asn	8	3.83 ± 0.04	−0.42 ± 0.06
Asp	8	3.02 ± 0.04	−1.23 ± 0.07[4]
	2	7.00 ± 0.03	0.07 ± 0.11[5]
Cys	8	4.49 ± 0.04	0.24 ± 0.06
Gln	8	3.67 ± 0.06	−0.58 ± 0.08
Glu	8	2.23 ± 0.10	−2.02 ± 0.11[4]
	2	6.94 ± 0.02	0.01 ± 0.15[5]
Gly	8	4.24 ± 0.02	−0.01 ± 0.05
	2	6.70 ± 0.02	
His	8	4.08 ± 0.02	−0.17 ± 0.06[5]
	2	5.97 ± 0.05	−0.96 ± 0.12[4]
Ile	8	4.52 ± 0.03	0.31 ± 0.06
Leu	8	4.81 ± 0.02	0.56 ± 0.04
Lys	8	3.77 ± 0.12	
	2	5.94 ± 0.02	−0.99 ± 0.11[4]
Met	8	4.48 ± 0.04	0.23 ± 0.06
Phe	8	5.38 ± 0.02	1.13 ± 0.05
Pro	8	3.80 ± 0.11	−0.45 ± 0.12
Ser	8	4.12 ± 0.07	−0.13 ± 0.08
	2	6.94 ± 0.02	
Thr	8	4.11 ± 0.03	−0.14 ± 0.06
	2	6.67 ± 0.03	
Trp	8	6.10 ± 0.02	1.85 ± 0.06
Tyr	8	5.19 ± 0.04	0.94 ± 0.06
Val	8	4.18 ± 0.02	−0.07 ± 0.05

[1]*Peptide synthesis and purification* have been described in detail elsewhere[7]. Purity of all peptides was greater than 99%, and the identity of all the peptides was confirmed by fast atom bombardment mass spectrometry. Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). The *buffer* used in all experiments was 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 3 mM NaN_3 at either pH 8 or pH 2. Large unilamellar vesicles (LUV) of approximately 100 nm diameter were made by extrusion[45] from palmitoyloleoylphosphatidylcholine (POPC). All lipids and peptides were found to be stable for the 24 hour duration of the partitioning experiments. Water-to-bilayer partition coefficients were measured using equilibrium dialysis and quantitative reverse phase HPLC and are expressed here in mole fraction units[15]. Partition coefficients were independent of pH between pH 7 and pH 9. All peptides have been shown to be monomeric and to lack well defined structure in aqueous solution[7]. Peptide partition coefficients did not depend on concentration, indicating that the peptides are monomeric in bilayers as well. This is corroborated by the observation that the CD spectra of membrane bound peptides (see below) are nearly identical to the random coil spectra in water. One exception is $AcWL_5$ which, at high concentrations in the membrane, has a CD spectrum that is consistent with a β-sheet structure and for which the partition coefficient increases dramatically at peptide per lipid ratios greater than 0.001. However the partition coefficient is independent of concentration below 0.001 peptides per lipid and the CD spectra indicate a random coil at these concentrations so even $AcWL_5$ appears to bind to membranes as unstructured monomers at low concentrations. Circular Dichroism measurements were made with a Jasco J720 CD spectrometer. Spectra were obtained from 20–100 μM peptide solutions placed in a 1 mm cuvette. Solutions also contained up to 4 mM POPC vesicles. Only AcWL-W-LL and $AcWL_5$ bind well enough for the spectra to be measured in POPC at pH 8. Some of the other peptides were examined at pH 2, where the binding is much better, but the solubility is much lower. In all cases examined except $AcWL_5$ at high concentrations (discussed above), the spectra of the membrane bound forms of the peptides were nearly identical to those of the soluble forms. All CD spectra, except for $AcWL_5$, were very similar in shape and ellipticity to that expected for a random coil peptide[46].

[2]Carboxyl group is fully ionized at pH 8 and fully protonated at pH 2. Free energies are based on mole-fraction units.

[3]Whole residue contribution to partitioning calculated as described in the text using Eq. 1. These values include the side chain and backbone contributions.

[4]Ionized side chain.

[5]Un-ionized side chain.

cating that the pH 8 values fall solidly on the upper plateau of a sigmoidal titration curve. We conclude that the C terminus-protonated forms of the peptides and the neutral forms of the acidic and basic side chains make no significant contribution to partitioning at pH 8.

The $\Delta G_X^{residue}$ values of Table 1 constitute an interfacial hydrophobicity scale that provides information on the principles governing the energetics of the interactions of peptides with lipid membranes. In addition, it should be useful for estimating the free energy of membrane partitioning of small oligopeptides that have a generally extended conformation. However, the scale must be considered as approximate for three reasons. First, the values are based on the sum of the backbone and the side-chain group contributions of a guest residue inserted between Leu neighbours. For smaller neighbours, the guest residues are likely to be apparently more hydrophobic because of smaller occlusion effects[7]. For example, the increase in the non-polar ASA (accessible surface area), ΔA_{np}, on insertion of a residue X into $AcWL_3$ to make AcWL-X-LL is smaller than the ΔA_{np} of that residue in a fully exposed state such as in G-X-G because the Trp and Leu neighbours occlude the ASA of the guest and because the guest occludes the ASA of the host[7]. Thus, the $\Delta G_X^{residue}$ values in Table 1 will be more hydrophobic in the context of smaller neighbouring side chains than the values listed. This occlusion is not a serious deficiency, however, because membrane-active peptides and proteins are rich in bulky side chains such as Leu. Second, the values of Table 1 are likely to depend on the chemical structure of the lipid headgroups which could affect peptide solvation and conformation, extent of association with the edge of the hydrocarbon region, and the so-called bilayer-effect contribution to partitioning[16]. Additional experiments with other lipid systems are needed to understand the influence of the headgroup on interfacial partitioning. Third, although the peptides appear to be random coils by CD spectroscopy, we cannot rule out the existence of differences in conformational restrictions in the water and bilayer phases that depend upon the guest residue. Other spectroscopic methods, such as NMR, may provide more detailed information about such differences. Despite its approximate nature, the pentapeptide-based scale provides significant information about peptide–bilayer interactions and serves as an important reference scale for future studies of other peptide and membrane systems.

The whole-residue free-energy values comprising our POPC-based interfacial hydrophobicity scale are summarized in a rank-order fashion in Fig. 2. The charged residues are at one end of the scale and the hydrophobes at the other end, indicating that the hydrophobic effect plays a central role in partitioning. A closer examination reveals several important points. First, interfacial partitioning of peptides will be dominated by the large favourable contributions of the aromatic residues and the large unfavourable contributions of the charged residues. Second, the next largest contributors are the carboxyamide side chains Gln and Asn which are unfavourable by ~0.5 kcal mol^{-1} and Leu, which is

favourable by ~0.5 kcal mol^{-1}. Third, the remaining residues make relatively small net contributions (~0.25 kcal mol^{-1}). Fourth, the interfacial hydrophobicities of all of the charged side chains are approximately equal, contrary to some expectations. Interfacial partitioning of Lys and Arg have been assumed to be relatively favourable because of the possibility of their methylenes interacting hydrophobically with the membrane interface while their charged moieties interact favourably with the aqueous environment[6,17,18]. The results of Fig. 2 indicate clearly that this so called 'snorkel effect' does not occur for the pentapeptides. This is consistent with the structure of a fluid phase phosphatidylcholine membrane[12] for which the membrane interface occupies a thickness of at least 15 Å, compared to the 6 Å length of an Arg and Lys side chains.

In octanol, the side chains of the Arg and Lys guests form intramolecular salt bridges with the C terminus of AcWL-X-LL and cause a free energy reduction of ~4 kcal mol^{-1} relative to a non-interacting charge pair[8]. Although we do not know if structurally equivalent interactions occur in the membrane interface, the data suggest the presence of weak, but favourable, electrostatic interactions. The Arg and Lys values of $\Delta G_X^{residue}$ in Table 1, –0.81 and –0.99 kcal mol^{-1} respectively, were determined from Eq. 1*b* using the pH 2 values of ΔG_{WLXLL} in order to eliminate the effect the C terminus charge. If Eq. 1*a* and the pH 8 values are used, $\Delta G_X^{residue}$ for Arg and Lys are found to be –0.34 and –0.44 kcal mol^{-1} respectively. A comparison of these values indicates that the charged C terminus may change the cost of partitioning Arg and Lys by $\Delta\Delta G \approx -0.5$ kcal mol^{-1}. However, this reduction must be judged against similar comparisons for the X=Gly, Ala, Ser, and Thr peptides used for the determination of ΔG_{COO} (see above) which may be due to pH-dependent changes in the peptide–bilayer interactions. Such comparisons yield $\Delta\Delta G$=–0.22, +0.18, +0.14, and –0.12 kcal mol^{-1} for the Gly, Ala, Ser, and Thr peptides respectively. These $\Delta\Delta G$ values vary in sign and have magnitudes that are significantly smaller than those for Arg and Lys. We thus conclude that there is probably a modest favourable interaction involving the Arg and Lys guests and the charged C terminus.

Aromatic residues have been proposed to have important roles in the structure and function in channel-forming peptides[19–21] and membrane proteins[16,22,23] because they are found with a high frequency near the ends of transmembrane domains that are expected to be in the membrane interface. Although the exact role of aromatic residues in membrane protein assembly and function is currently unknown, the pentapeptides with aromatic guest residues also have a strong interaction with the membrane interface (Fig 2). As we discuss below, the high interfacial hydrophobicity of the aromatics is disproportionally large relative to their hydrophobicity in octanol. This agrees with studies of the exceptionally strong partitioning of indole and indole analogues into the membrane interface[16] and strengthens the idea that there are special interactions there with these groups.

How does our experimentally determined interfacial scale (Table 1) compare to scales such as those of Kyte and Doolittle[24] (KD) and Engelman, Steitz and Goldman[25] (GES) commonly used in hydrophobicity plots? A strict comparison is difficult because both the KD and GES scales involve adjustments of various experimental values based on guesses about which properties of amino acids may be particularly important in non-polar environments. The aromatic residues are good examples. Both KD and GES place Phe among the upper one-third (most hydrophobic) of the amino acids in company with Leu, Ile, and Val and place Trp and Tyr in the middle-third along with Ser, Pro, and Thr. These placements mainly reflect the presence or absence of explicit polar moieties and assumptions about the hydrogen bonding of the moieties in non-polar environments. Our experimental scale is unambiguous about the placement of the aromatic residues: they are the most hydrophobic. Although we have speculated that specific dipole-dipole interactions in the bilayer headgroup region may be important[16], a physical basis for the aromatics' dominant position is lacking.

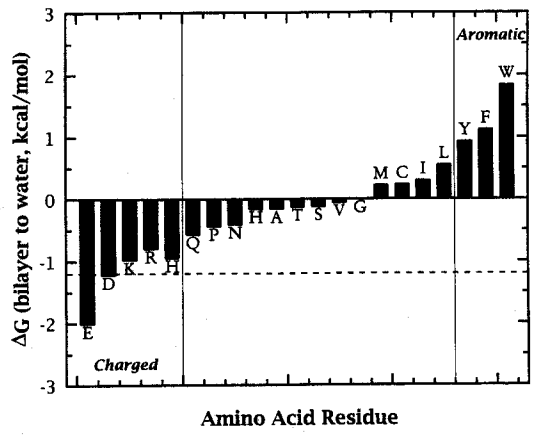

Fig. 2 Rank-ordered whole-residue interfacial hydrophobicity for the 20 natural amino acids calculated as described in the text from the partitioning of AcWL$_m$ and AcWL-X-LL. The values are listed in Table 1. Note that the charged residues and the aromatic residues dominate the whole residue contributions. The horizontal dashed line is the estimated contribution of the peptide bond to interfacial hydrophobicity (see text).

Peptide bond interfacial hydrophobicity

One can use the solvation parameter formalism of Eisenberg and his colleagues[26] to estimate the contribution of the Leu side chain to the whole-residue free energy. The apparent non-polar solvation parameter $\Delta\sigma_{np}^{Leu}$ for the Leu side chain relative to Ala is given by:

$$\Delta\sigma_{np}^{Leu} = \frac{\Delta G_{WLLLL} - \Delta G_{WLALL}}{A_{Tnp}^{WLLLL} - A_{Tnp}^{WLALL}}$$

where the total non-polar accessible surface areas A_{Tnp}^{WLXLL} of the peptides are those determined by Monte Carlo simulations[7]. This calculation yields a value $\Delta\sigma_{np}^{Leu}$ =13.1±0.6 cal mol^{-1} Å^{-2} which is consistent with the non-polar solvation parameter of 12 cal^{-1} mol^{-1} Å^{-2} determined from the partitioning of five hydrophobic tripeptides[6]. These values are about one-half the value determined for octanol partitioning[7]. As we show below,

the free energy contributions of many other charged, polar and non-polar groups to interfacial partitioning are also approximately one-half the values for octanol.

The peptide bond contribution to partitioning ΔG_{CONH} can be estimated from the incremental free energy change $\Delta\Delta G_{Leu}$ (see above) by subtracting the side chain plus Cα hydrophobic free energies using:

$$\Delta G_{CONH} = \Delta\Delta G_{Leu} - \Delta\sigma_{np}^{Leu} \cdot \Delta A_{np}^{Leu}$$

where ΔA_{np}^{Leu}, the total non-polar accessible surface area of the Leu side chain and Cα, is[7] 134 Å^2 determined by plotting the total non-polar accessible surface areas of the AcWL$_m$ peptides against m. This calculation yields ΔG_{CONH} = –1.2±0.1 kcal mol^{-1} or about one-half the value of –2.0 kcal mol^{-1} observed for partitioning from octanol[7].

The peptide bond is much more polar than any of the uncharged polar side chains. This is emphasized by the free energies of transfer for structurally similar Asn and Gln which, relative to Ala, are –0.25 and –0.41 kcal mol^{-1}, respectively. A comparison of ΔG_{CONH} with the whole-residue values of Table 1 shows that it is a major determinant of amino acid partitioning free energies. Of particular significance is the likelihood that the water-to-membrane transfer free energy of a hydrogen-bonded peptide bond is smaller than that of a non-hydrogen bonded one[14,27]. Thus, interfacial hydrophobicities of most of the uncharged residues should become much more favourable if the peptide bond participates in hydrogen bonded secondary structure. We speculate that the free energy loss associated with hydrogen bond formation may be an important factor in secondary structure formation at membrane interfaces.

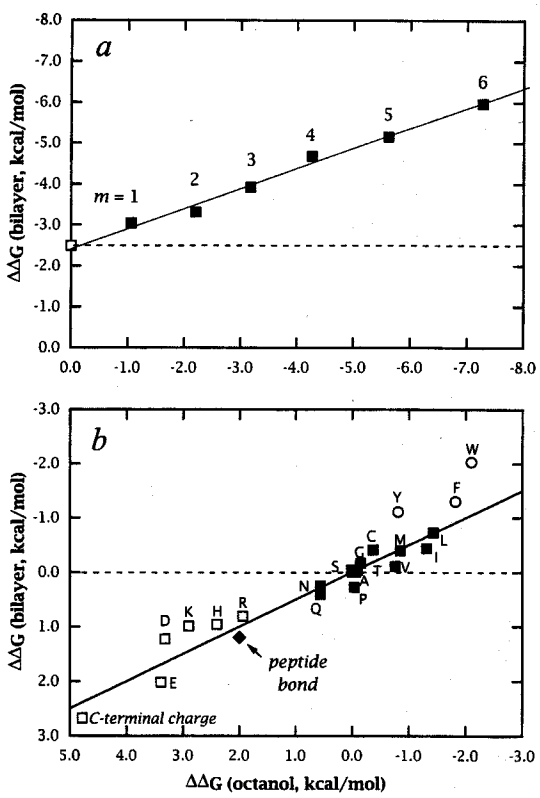

Fig. 3 Comparisons of water-to-bilayer partitioning with water-to-octanol partitioning for AcWL$_m$ and AcWL-X-LL. All free energies are in kcal mol^{-1} calculated using mole-fraction units. Experimental uncertainties are approximately the diameters of the data points. *a*, The bilayer and octanol data for AcWL$_m$ are from Table 2. The calculated point for AcW-OOH (□) was obtained by subtracting the free energy of transfer of one Leu residue and the C-terminal charge from ΔG for AcWL. All values are taken relative to AcW-OOH in octanol. The solid line is the result of a linear regression fit of the bilayer data to the octanol data. The slope is 0.49±0.04 and the intercept –2.40±0.16 kcal mol^{-1}. *b*, Free energies of transfer of the side chains relative to AcWL-A-LL, the C terminus, and the peptide bond. The solid line has the slope of 0.49 determined in (*a*).

Comparison with octanol partitioning

We compare directly in Fig. 3 the octanol and membrane partitioning of the two families of peptides. Fig. 3*a* shows that the partitioning of AcWL$_m$ into bilayers is a linear function of octanol partitioning with an intercept of –2.4 kcal mol^{-1} and a slope of 0.49. The length-independent negative intercept suggests that the entropy cost for immobilizing a peptide into the membrane interface may not be as large as some estimates[28] which is consistent with a recent statistical mechanical analysis of peptide-membrane interactions[29] and measurements of the bilayer partitioning of fatty acids[30]. The slope of 0.49 means that the net contribution of a Leu to the free energy of membrane partitioning is about one half of the value in octanol. Fig. 3*b* shows that a slope of 0.5 also roughly describes the relative contributions of the side chain, C terminus, and peptide bond groups. The deviations from the line (Fig. 3*b*) reveal interesting differences between bilayer and octanol partitioning. For example, the β-branched hydrophobes Val and Ile are much less hydrophobic in the bilayer than expected whereas the aromatics are more hydrophobic. The observation that interfacial hydrophobicity is proportional to octanol hydrophobicity supports the earlier conclusion that membrane partitioning is driven mainly by the hydrophobic effect[6]. However, the slope of 0.5 of the solid line and the fluctuations of the points around it reveal that the details of the interactions with the membrane-water interface contribute important effects.

Jacobs and White[6] offered two possible explanations for the observation that the interfacial non-polar solvation parameter of their five tripeptides was about one-half the value expected for partitioning into bulk organic phases: either the smaller solvation parameter is a general property of the complex interfacial region or the non-polar surface is ~50% buried in the outer edge of the hydrocarbon core. The data presented here suggest that the first explanation is more likely because the solvation parameters of charged, polar, and non-polar moieties alike are about one-half the values observed for octanol partitioning. This view is consistent with the structural image of the bilayer interface obtained by combined X-ray and neutron diffraction measurements[12]. That image shows that the 15 Å thick interface, defined as the region occupied by the waters of hydration, contains portions of the hydrocarbon core methylenes and the glycerol, phosphate, and choline groups. The high thermal disorder of the region suggests a com-

plex mixture of these diverse chemical entities. A simple, but probably incomplete, explanation of the 50% effect is that the average dielectric coefficient (ε) of the interface is slightly higher than that of octanol. For example, based on the calculations of Flewelling and Hubbell[31], an increase of ε from about 12 in hydrated octanol to 18 in the bilayer interface would explain the reduction of ΔG_{COO} from 4.78 to 2.68 kcal mol^{-1}. However, because structural studies[32] of octanol indicate that it too is a complex interfacial phase, simple explanations based solely on bulk-phase properties are probably not warranted.

Comparison with other experimental scales

While there are several computationally derived membrane hydrophobicity scales[6,25,33,34], we are not aware of any other experimentally determined scales that include all twenty natural amino acids. There are, however, two studies that provide membrane hydrophobicities for limited numbers of amino acids. The first is that of Jacobs and White[6] using A-X-A-*O*-*t*-Bu with X=G,A,L,F,W. The magnitudes of the side-chain values are about the same as for the pentapeptides and increase in the order Gly<Ala<Leu<Phe<Trp. The other host-guest study is the recent one of Thorgeirsson *et al.*[35] who examined 14 of the natural amino acids in guest positions in either of two 25-residue analogues of the presequence of subunit IV of cytochrome C oxidase (COX IV). They studied binding to LUVs formed from a mixture of POPC and negatively charged palmitoyloleoylphosphatidylglycerol (POPG). The peptides bind mainly through electrostatic interactions which were accounted for by the Gouy–Chapman–Stern theory[36] to obtain a set of amino acid side-chain free energies. The amino acid contributions in the COX IV analogies are proportional to octanol hydrophobicity but differ from the pentapeptide results in two significant ways. First, the bilayer and octanol hydrophobicities are related by a slope of approximately 1. Second, the magnitudes and rank ordering of the most hydrophobic residues in the COX IV peptides are Tyr<Leu~Phe~Trp whereas for the pentapeptides they are Leu<Tyr<Phe<Trp.

The reasons for these differences between the pentapeptide and COX IV scales are not clear, but there are several possibilities. First, the pentapeptides were partitioned into pure POPC bilayers and the COX IV peptides into POPC:POPG bilayers. The differences may thus reflect differences in interfacial solvation parameters. Second, the COX IV peptides bind primarily through electrostatic interactions. This could cause the peptides to have a different disposition in the membrane interface. Third, the COX IV peptides are significantly larger so that the perturbations of the bilayer induced by the two classes of peptides are likely to be quite different. This difference could result in differences in the bilayer-effect contribution to the free energy of partitioning[16].

Despite a huge literature concerned with the interactions of a wide range of peptides with membranes, there are surprisingly few measurements of phosphatidylcholine partition coefficients that are suitable for comparing the predictions of the pentapeptide and COX IV scales. Replacement of all five of the tryptophans in the 13-residue antimicrobial peptide[37,38] indolicidin (IND) with Phe, Tyr, or Leu gives POPC partition coefficients that are ranked in the order IND_{Leu} ~ (IND_{Tyr}<<IND_{Phe}<<IND_{Trp} (A.S. Ladokhin and S.H. White, unpublished data). These observed rankings and magnitudes are approximately as predicted by the pentapeptide scale, while they are different by up to several orders of magnitude from the changes (Tyr<<Leu~Phe~Trp) predicted using the COX IV scale. Tretyachenko-Ladokhina *et al.*[39] mutated two of the tryptophans in the membrane binding domain of cytochrome b5 to leucine and found that the binding decreased by about 2.2 kcal mol^{-1}, consistent with the expectations of the pentapeptide scale. Similarly, Nakajima *et al.*[40] changed Ile 8 and Leu 12 of dynorphin to Phe and found that the binding constant increased by 0.9 kcal mol^{-1}, again consistent with the pentapeptide scale.

Table 2 Free energies of transfer of $AcWL_m$ peptides to water

m	ΔG from bilayer[1] (kcal mol^{-1} ± SEM)	ΔG from octanol[2] (kcal mol^{-1} ± SEM)
1	3.17±0.02	–0.92±0.05
2	3.44±0.03	0.22±0.04
3	4.05±0.03	1.19±0.01
4	4.81±0.02	2.29±0.01
5	5.28±0.02	3.64±0.02
6	6.10±0.39[3]	5.30±0.03[3]

[1]Free energies determined at pH 8.0 using mole fraction units.
[2]Free energies determined at pH 9.0 using mole fraction units[7].
[3]The $AcWL_6$ peptide appears to aggregate somewhat in the aqueous phase. For this reason, its values of ΔG were not used in any computations.

Conclusions

Elucidation of the principles of membrane protein folding and insertion logically begins with the fundamental physicochemical principles of peptide–membrane interactions. The properties of amino acids that determine their secondary structure propensities in membrane environments have been examined recently by Deber and his colleagues[41,42] and the basic principles of electrostatic interactions at bilayer interfaces by Thorgeirsson *et al.*[36] and Ben-Tal *et al.*[43]. The present work provides a complete hydrophobicity scale for the partitioning of amino acid residues into electrically neutral (zwitterionic) membrane interfaces. In addition, it supports recent theoretical findings[29] which suggest that the mass-dependent free energy cost of bilayer partitioning is smaller than predicted by earlier analyses[28]. Engelman and his colleagues[25,44] suggested a number of years ago that the reduction of the free energy cost of partitioning peptide bonds brought about by intramolecular hydrogen bond formation is essential for partitioning proteins into and across the membrane hydrocarbon core. Recent electrostatic calculations support and elaborate on that suggestion[27]. The high cost of partitioning peptide bonds into the membrane interface, established by the results reported here, indicate that those considerations also apply to proteins in the bilayer interface.

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