Chapter 2

Energetics of Peptide and Protein Binding to Lipid Membranes

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Abstract

In every living cell, the lipid bilayer membrane is the ultimate boundary between the contents of the cell and the rest of universe. A single breach in this critical barrier is lethal. For this reason, the bilayer's permeability barrier is the point of attack of many offensive and defensive molecules, including peptides and proteins. Depending on one's perspective, these pore-forming molecules might be called toxins, venoms, antibiotics or host defense molecules and they can function by many different mechanisms, but they share one feature in common: they must bind to membranes to exert their effects. The thermodynamic and structural principles of polypeptide-membrane interactions are described in this chapter.

The Lipid Bilayer Phase

The hydrocarbon core of an unperturbed lipid bilayer membrane is one of the most hydrophobic microenvironments found in nature, with water concentration, dielectric constant and charge density that are very similar to an alkane phase in equilibrium with water. The hydrophobicity of the bilayer core dominates the membrane interactions of many classes of molecules; from ions and drugs to peptides and proteins.[1] Yet, as little as one nanometer away from the truly nonpolar core, the bilayer membrane contains an interfacial zone rich in polar groups, including water, as well as lipid hydrophobic moieties.[1-3] This broad interfacial region contains a sharp gradient of polarity, forming an anisotropic transition zone between the polar aqueous phase and headgroup region and the nonpolar bilayer core. As shown in Figure 1 the lipid bilayer membrane can be represented by three distinct zones of equal total thickness/volume: the hydrocarbon core, bounded on either side by a broad interfacial zone.

The hydrocarbon core of the membrane imparts a strict barrier to the permeation of most polar or charged solutes through the bilayer. Operationally, a "pore-forming" molecule can be defined as one that increases the permeability of a bilayer to polar solutes. There are at least two fundamentally different mechanisms by which a peptide or protein can alter membrane permeability: (1) A molecule can *work with* the hydrocarbon core by utilizing its constraints to drive self-assembly or folding of a polypeptide into a specific three dimensional structure, such as a protein pore, that provides a mostly protein polar channel or pathway through the membrane. This mechanism requires a membrane protein-like match between the hydrophobicity profile of the bilayer and the hydrophobicity profile of the inserted molecule. (2) Alternately, a molecule can *work against* the hydrocarbon core by altering the lipid packing and organization such that a mostly-lipid pathway through the lipids is created that eliminates the requirement for a polar solute to pass through a nonpolar layer. Molecules with this type of activity are amphipathic, but not perfectly amphipathic,

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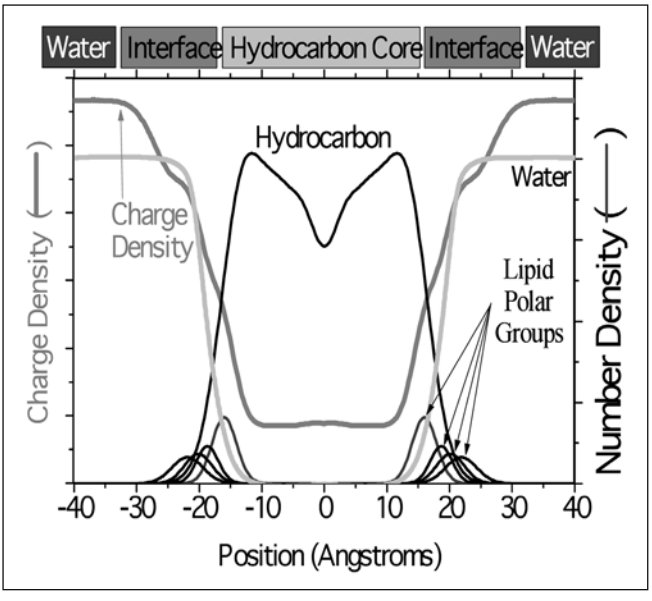

Figure 1. The lipid bilayer membrane. Depth profiles across an unperturbed lipid bilayer membrane. These are experimentally measured distributions of hydrocarbon and polar groups, including water, across a lipid bilayer membrane.[1] The center of the hydrocarbon core is assigned a position of 0 Å. Note that the nonpolar core of the membrane is less than 30 Å wide and is bounded on both sides with an broad anisotropic interfacial zone that contains hydrocarbon, polar groups and water. The charge density profile denotes the polarity gradient across the bilayer. Given the complexities of the bilayer physical chemistry, many types of interactions can take place.

such that the bilayer must be deformed and the hydrocarbon core disrupted (i.e., intermingled with lipid polar groups) to simultaneously accommodate the polar and charged moieties of the polypeptide. Independent of the mechanism, pore-forming peptides and proteins must interact strongly with membranes and that is the subject of this chapter.

Hydrophobic Interactions

Any pore-forming peptide and/or protein must interact more favorably with membranes than with either water or themselves in water. Binding is driven by hydrophobic and electrostatic interactions. Conceptually, it has been useful to consider membrane binding, self-assembly and folding as separate steps linked by thermodynamic cycles. For example Popot and Engelman described a two-state model for insertion and folding of membrane proteins[4] which was augmented by Wimley and White[1,5] into a four step cycle comprising interfacial partitioning, folding, insertion and assembly. This four step model is especially appropriate as a foundation to describe the interactions of pore-forming peptides and proteins with membranes because many of them actually follow such a pathway.

Partitioning of polypeptides from water to membranes is often dominated by hydrophobic interactions. To understand or predict interactions with the complex and anisotropic bilayer one must begin with quantitative measure of the propensity of a polypeptide to physically associate with a membrane. The Wimley-White interfacial hydrophobicity scale and octanol partitioning scale are hydrophobicity scales which have been shown to be useful to understand binding, insertion and folding of polypeptides in membranes.[1,6-9] These are experimentally-determined, whole residue scales that include the cost of partitioning the peptide backbone and thus give absolute free

energies that can be used for predictions and experimental design. These scales also give information on the energetics of folding and how it is coupled to partitioning. The free energy values for the interfacial scale, shown in Figure 2, represent the free energy of partitioning of unstructured peptides into the fluid phase phosphatidylcholine bilayer interface. Only six amino acids are significantly favorable for partitioning into bilayers in the context of a random coil peptide: the aromatic residues: tryptophan, tyrosine and phenylalanine; and the aliphatic residues: methionine, leucine and isoleucine. The aromatic residues make especially large contributions and essentially dominate the interactions of peptides with membrane interfaces. In fact, it is unusual to find a membrane-partitioning polypeptide that does not have at least several aromatic amino acids. The charged amino acids are the only ones that strongly oppose partitioning into the interfacial region of the bilayer, although the energies of only 1-2 kcal/mol are not nearly as large as they were once thought to be. These residues remain fully ionized in bilayers.[6]

The octanol hydrophobicity scale is based on measurements of peptide partitioning into the more nonpolar environment of a hydrated octanol phase. This scale has been shown to be relevant to proteins inserted into the bilayer hydrocarbon core. In fact it allows for very accurate prediction of membranes-spanning segments of membrane proteins.[10] Although hydrated octanol is more polar than the core of an unperturbed bilayer, it must be similar to the local environment experienced by a polypeptide and its associated polar groups in the hydrocarbon core. In the octanol hydrophobicity scale free energy values are roughly double the values for the interfacial scale, except for the aromatics which have a special interaction with bilayer interfaces.[11]

Based on these hydrophobicity scale data, shown in Figure 2, one can calculate the contribution of hydrophobicity to membrane partitioning and predict polypeptide segments likely to interact with and insert into membranes. If we use the original mole fraction units defined as

Mole-Fraction Partition Coefficient

$$Kx = x_{\text{bilayer}}/x_{\text{water}}$$

X_{bilayer} = [peptide] in bilayer/[lipid] in bilayer

X_{water} = [Peptide] in water/[water] in water (55.3 M at R.T.)

$$\Delta G_x^0 = -RT \ln K_x$$

then the total free energy of hydrophobic partitioning of a polypeptide can be written as the sum of the whole residue contributions, shown in Figure 2, plus the sum of the contributions from the termini.[9]

$$\Delta G_X^{\text{Interface}} = \left(\sum \Delta G_X^{\text{residue}}\right) + \Delta G_X^{\text{N terminus}} + \Delta G_X^{\text{C terminus}}$$

Mole fraction free energies of binding in the range of −5 to −12 kcal/mol are typical for pore-forming peptides and proteins. In practical terms, mole fraction partition coefficients can be used to calculate the fraction of peptide that is membrane bound as a function of lipid concentration,

$$\text{Fraction of peptide bound} = K_X[L]/(K_X[L] + 55.3\ \text{M})$$

where K_x is the mole fraction partition coefficient, $[L]$ is the molar concentration of lipid and 55.3 is the molar concentration of water. A water-to-bilayer ΔG_x of −4 kcal/mol (favorable) is equal to $K_x = 860$, a partition coefficient that describes a peptide which is less than 2% bound at 1 mM lipid concentration. This level of binding is near the lower limit of detectability and in most experimental systems would not be able to drive membrane permeabilization. A ΔG_x of −10 kcal/mol provides for a very strong interaction in which greater than 99% of peptide is bound at 1 mM lipid. It is difficult to design a hydrophobic peptide with ΔG_x more favorable than about −12 kcal/mol, because loss of peptide solubility makes such extremely hydrophobic peptides very difficult to use. These limits set the range of useful hydrophobic partition coefficients that are consistent with the function of pore-forming polypeptides.

Amino Acid	*Interface Scale* (kcal/mol)	*Octanol Scale* (kcal/mol)
Ala	0.17	0.50
Arg^+	0.81	1.81
Asn	0.42	0.85
Asp^-	1.23	3.64
Asp^0	−0.07	0.43
Cys	−0.24	−0.02
Gln	0.58	0.77
Glu^-	2.02	3.63
Glu^0	-0.01	0.11
Gly	0.01	1.15
His^+	0.96	2.33
His^0	0.17	0.11
Ile	−0.31	−1.12
Leu	−0.56	−1.25
Lys^+	0.99	2.80
Met	−0.23	−0.67
Phe	−1.13	−1.71
Pro	0.45	0.14
Ser	0.13	0.46
Thr	0.14	0.25
Trp	−1.85	−2.09
Tyr	−0.94	−0.71
Val	0.07	−0.46

Terminal group	*Interfacial Scale* (kcal/mol)
$+H_3N$-	0.48
H_2N-	-0.02
Acetyl-	0.10
-COO-	-0.75
-COOH	-3.43
$-CONH_2$	-2.65
Residues with structure	-0.4/residue

Figure 2. Hydrophobicity scales. Whole-residue, mole-fraction free energy values for peptide partitioning into bilayer interfaces or into hydrated octanol from water. These experimentally determined hydrophobicity scales are described in detail elsewhere.[1,6,22] The signs have been reversed relative to the original publications to reflect free energies of transfer *from* the water phase, thus a negative ΔG is an interaction that favors partitioning. Determination of the free energy of the termini are described in.[9] The carboxyl terminal value contains an additional entropic term possibly related to the reduction in dimensionality upon binding. The per residue decrease in ΔG for folding is also experimentally determined. This is an average value that could vary between peptides. Because these are experimentally determined, whole residue values, a prediction of the free energy of hydrophobic partitioning of any peptide can be made from a simple summation of the values, as described in the text.

Electrostatic Interactions

The other major driving force for polypeptide-membrane partitioning is electrostatic interaction. The charged moieties of lipid bilayers are found in the outer-most part of the interfacial zone, comprising the lipid headgroup moieties along with a high concentration of water and other polar groups (Fig. 1). This double-layer of concentrated surface charge can drive strong electrostatic interactions between bilayers and polypeptides. Biological membranes are composed of mixtures of neutral lipids, zwitterionic lipids and anionic lipids. Cationic lipids are extremely rare in nature. Thus biological membranes are often anionic; and membrane-interacting polypeptides are almost always cationic. This is especially true for the small membrane-active peptides such as the lytic toxins or antimicrobial peptides in which net charges can be as high as +10 can be found.

Electrostatic interactions are long-range and can guide polypeptides to a membrane surface where very high interaction free energies can result. Using Figure 1 as a guide, one can consider a polypeptide that has favorable electrostatic and favorable hydrophobic contributions to membrane binding. As a peptide approaches the bilayer surface electrostatic interactions increase rapidly and reach a maximum in the vicinity of the phosphate groups, which reside on the outermost portion of the interfacial zone. In terms of mole fraction partitioning, energies as high as −10 kcal/mol can result from electrostatic interactions under physiological ionic strength and modest surface charge on the bilayers and charge on the peptide. Calculation of electrostatic interactions has been described by Murray.[12]

A universal feature of systems with charged polypeptides binding to bilayers is anti-cooperative binding.[5] This occurs because the net charge on the bilayer surface is reduced by polypeptide binding and also because the bilayer-accumulated charged peptides disfavor additional binding due to repulsive interactions. Seelig and others have developed methods to deconvolute such contributions to bilayer interactions.[13] A consequence of anti-cooperative binding is that partitioning experiments will appear to give a saturable binding curve, which one can fit with a classical binding site model. However, such models are inappropriate to describe peptides partitioning into membranes.[5]

Additivity between Electrostatic and Hydrophobic Interactions

At the depth in the bilayer interface where electrostatic interactions are strongest, hydrophobic interactions are weak because the polarity and water content near the charged headgroup moieties are close to the bulk water values. Hydrophobic interactions will become significant only as a polypeptide partitions deeper into the membrane, away from bulk water phase. However this occurs *at the expense* of electrostatic interactions, which decrease as a peptide moves away from the headgroup region of the interface. The equilibrium depth of insertion will depend on the balance of the two interactions and on the ability of the lipids and peptide chain to deform to accommodate them. Strong electrostatic binding, without a hydrophobic component is generally not sufficient to perturb the hydrocarbon core because electrostatically bound polypeptides are bound only to the surface. Importantly, the dissimilar depth profiles for electrostatic and hydrophobic interactions means that free energies will not be additive, which has been shown experimentally.[14]

The dissimilar depth profiles of the hydrophobic and polar/charged moieties of the lipid bilayer lends itself to disruption of the hydrophobic core by imperfectly amphipathic polypeptides such as the antimicrobial peptides, because these molecules drive the mixing of polar and charged residues with the hydrophobic core, leading to a situation where the interactions can only be satisfied simultaneously by a highly perturbed bilayer.

The Influence of Peptide and Protein Structure

An open peptide bond is one of the most polar moieties in a polypeptide chain, costing as much to partition into a bilayer as some of the charged side chains. The cost is about 1.2 kcal/mol per residue in the interface and about 2 kcal/mol in the hydrocarbon core. Because the cost of partitioning a hydrogen-bonded peptide bond is lower, there will always be a strong driving force for folding that is coupled to partitioning into a bilayer. Based on various experiments with β-sheet and α-helical peptides, the net free energy change for folding in a bilayer has an average value of about −0.4 kcal/mol/residue (range: −0.2 to −0.5 kcal/mol). The consequence of this effect is that partitioning and folding are tightly coupled and peptides that have partitioned into bilayers will have a dramatically greater propensity for structure in the membrane that in solution. For example, the 22 residue pore-forming peptide melittin is calculated to have a mole fraction partitioning free energy as a random coil of −1 kcal/mol, a value that denotes such weak binding that it is not measurable. In reality, melittin binds very strongly with a ΔG_x of −8 kcal/mol. Strong binding comes about because the weak random coil binding is coupled to a contribution of about −0.4 kcal/mol/residue of folding for each the 15-18 residues that change from random coil to α-helix upon membrane partitioning.[15]

Specific Interactions

Pore-forming peptides and proteins that partition into membranes because of hydrophobic interactions will interact with almost any bilayer type. Classical examples of this nonspecific behavior are the α-helical pore-forming peptides melittin and alamethicin and the beta-helix gramicidin A. These pore formers will permeabilize almost any fluid phase bilayer membrane, in a living cell or in a test tube. Some cationic pore-forming peptides, especially the antimicrobial peptides, target anionic membranes specifically by partitioning into them preferentially due to a strong electrostatic component. Nonetheless, peptide-membrane interactions that occur by partitioning are, by definition, relatively nonspecific and will require more than the minimum number of residues to drive the interaction. For example, a minimum of several aromatics or aromatics mixed with aliphatic residues are required for moderately good hydrophobic partitioning.

There are many pore-forming proteins and peptides that interact with membranes by highly specific interactions. For example, diphtheria toxin as well as the pore-forming colicins are targeted to membranes by a highly specific receptor-protein like interaction. Similarly, the antibiotic peptides vancomycin and the type A lantibiotiocs, such as nisin, have highly specific interactions with particular lipid components of bacterial membranes, which they subsequently permeabilize. The cholesterol-dependent cytolysins are pore-forming proteins with a very strict requirement for cholesterol in the target membranes. All these examples of pore-forming polypeptides coupled to strong and highly specific interactions with individual components of the membrane should probably be treated like a ligand-binding-pocket type of interaction rather than an interaction that is dependent on partitioning. In any case, once a pore-forming peptide or protein has interacted with a target membrane, the membrane is subsequently permeabilized by one of the mechanisms described next.

Specificity: The Formation of Ordered Pores

The three dimensional structure of the *Staphylococcus aureus* α-hemolysin (Fig. 4) is a stunning example of a classical pore-forming protein. However the literature suggests that a stable,

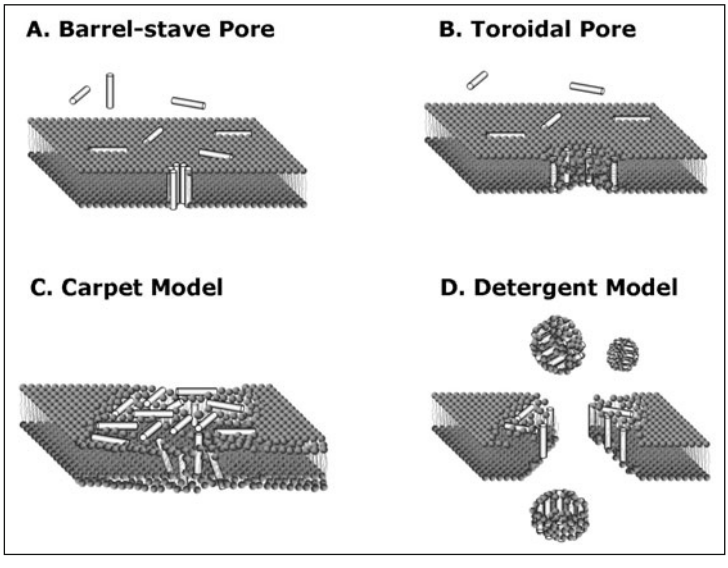

Figure 3. Some models of polypeptide membrane permeabilization. Some mechanistic models of membrane permeabilization by polypeptides. There are many different mechanisms by which membrane permeabilizing peptides and proteins can function. Some of the more commonly described mechanisms are shown in these schematic images. The driving forces and implications for these various models are described in the text.

well structured protein pore with a fixed stoichiometry like α-hemolysin is actually a very rare counter-example to the majority of known pore-forming polypeptides, which number well over 1000. Most do not form such fixed structures but rather bind to membrane surfaces through specific or nonspecific interactions and then self-assemble into flexible, transient or flickering structures which allow permeation of solutes through the membrane. The simplest models of membrane permeation by polypeptides involve the formation of transbilayer pores or channels through the membrane as shown by the models in Figure 3. In a barrel stave pore, peptides interact laterally with one another to form a specific folded structure that is reminiscent of a membrane protein ion channel. In the toroidal pore model, specific peptide-peptide interactions are not present. Instead peptides affect the local curvature of the bilayer in a cooperative manner such that a toroid of high curvature forms through the bilayer. In either case, one can imagine pores that are stable and long lived or pores that are transient in equilibrium with surface bound or monomeric peptide. In fact only a very small fraction of the total peptide need be in a pore state at any moment in time to drive observed rates of leakage through membranes.

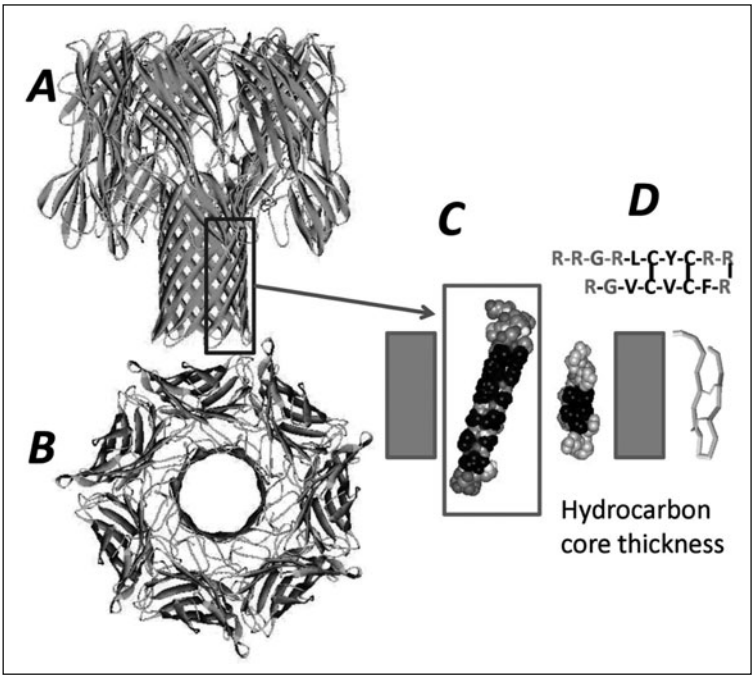

Figure 4. Transbilayer profiles of some pore-forming polypeptides. The structure of the protein α-hemolysin from Staphylococcus aureus.[23] A) This classical protein pore assembles into a heptameric ring on susceptible membranes which inserts a β-sheet, barrel stave pore across the membrane. B) The top view shows a distinct open pore through the protein (and through the membrane) which allows unrestricted leakage through the membrane and cell lysis. C) The lipid-facing surface of one of the β-hairpins from α-hemolysin. The thickness of the hydrophobic face (nonpolar residues in black) matches the profile of the hydrocarbon core of the membrane. This is why the barrel stave pore can assemble into an ordered pore in membranes. D) In sharp contrast, protegrin 1, a porcine antimicrobial peptide with a similar β-hairpin secondary structure has a hydrophobic face (in black) that is far smaller than the bilayer hydrocarbon core and is bounded by basic arginine residues. Protegrin binds strongly to bilayer through a combination of hydrophobic and electrostatic interactions. However, instead of forming a transbilayer pore, protegrin disturbs the lipid packing through its interfacial activity and imperfect amphipathicity.

Barrel stave and toroidal pores are functionally similar, but are fundamentally different in structure and membrane interactions. For example barrel stave pores work with the bilayer hydrocarbon core, using it as a template for amphipathic peptide-self assembly. Specific interactions between amino acids also contribute to self-assembly of the pore. Toroidal pores, on the other hand, work against the hydrocarbon core, disrupting the natural segregation of polar and nonpolar parts of the membrane by providing alternative surfaces for lipid to interact favorably with. Toroidal pores are formed by imperfectly amphipathic peptides.

Protein pore formers can form stable long-lived pores or flickering transient pores. Diptheria toxin and the pore-forming colicins, for example, can form transient pores across membranes by inserting interfacially-bound amphipathic helices across the membrane subsequent to the initial binding events. Peptides as well can form barrel stave or toroidal pores,[16] although distinguishing them from each other is not straightforward. A classical example of peptide that forms transmembrane pores is alamethicin, which forms an amphipathic alpha helix that can exist, depending on hydration and concentration, either mostly parallel or mostly perpendicular to the lipid bilayer normal.[16] The perpendicular structure is consistent with the image of a transmembrane pore and other evidence suggests a barrel stave pore for alamethicin.

Promiscuity: Membrane-Permeabilization by Interfacial Activity

In addition to the long held models of transmembrane barrel-stave or toroidal pores, a number of nonpore models have been proposed to explain or categorize the mechanism of pore-forming polypeptides in lipid membranes. The mechanism of action of the antimicrobial peptides has been especially difficult to explain with specific pore models. The so called "carpet model" is the most commonly cited phenomenological model and was proposed in 1996 by Shai[17] to explain the mechanism of action of mammalian cecropin P1 on model membranes. Cecropin P1 is a helical peptide that is oriented parallel to the membrane surface and does not form explicit pores. The peptide is active only at high P:L ratios, conditions under which the peptide forms a carpet on the bilayer surface. The "detergent model" is also often cited to explain the catastrophic collapse of membrane integrity observed with some anti microbial peptides at high peptide concentration leading to size-independent, partial leakage of entrapped contents.[18,19]

The majority of known membrane permeabilizing peptides are antimicrobial peptides and most of these function by a mechanism that is consistent with a nonspecific mechanism of membrane permeabilization.[20] This nonspecific activity has been described as "interfacial activity"[20] and is dependent on the ability of a peptide to bind to the membrane interface with hydrophobic and electrostatic interactions, followed by perturbation of the bilayer lipid packing driven by the broken, or imperfect amphipathic nature of the peptide (and bilayer). Marrink and colleagues have simulated such systems resulting in a very compelling image of a "pore-forming" antimicrobial peptide (magainin) that permeabilizes membranes by perturbing the bilayer's lipid packing and organization enough to break down the segregation between interface and core.[21] This breakdown allows permeation of polar molecules and does so without the formation of a transmembrane "pore" or channel. A realistic image of a peptide "pore" based on the studies of Marrink is shown in Figure 5.

Conclusion

Decades of experiments and modeling of pore-forming proteins and have shown that there are many different ways for a membrane to be permeabilized. These mechanisms range from highly specific, stable pore formation to nonspecific detergent-like membrane disruption. All of these mechanisms occur in nature and have biological relevance. However different the mechanisms, all pore-forming proteins and peptides must interact with membranes through binding, partitioning or a combination of the two followed by the formation of a polypeptide-induced polar pathway through the hydrocarbon core of the membrane. In this

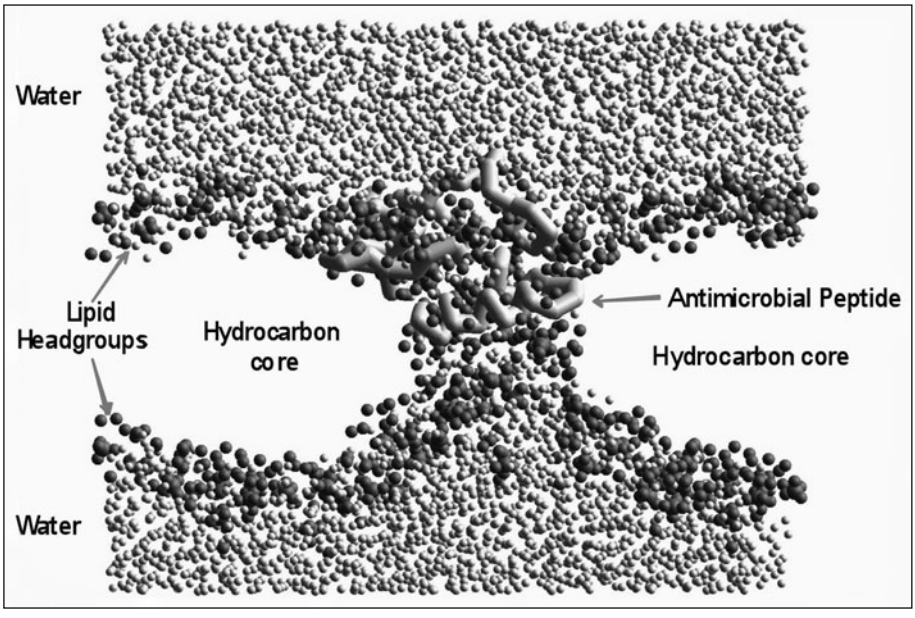

Figure 5. A simulated antimicrobial peptide in membranes. A realistic image of a peptide-induced pore in a bilayer. This image was adopted from the work of Marrink and colleagues[21] who simulated the interaction of an antimicrobial peptide, magainin, with phospholipid bilayers. Instead of forming a regular, ordered channel, this "pore-forming" peptide acts by using its imperfect amphipathicity to disrupt the segregation of polar and nonpolar parts of the membrane. Notice the high concentration of polar groups infiltrating the nonpolar core in the vicinity of the peptides, shown as tubes. Waters are shown as light gray spheres while lipid polar groups (phosphate, choline and the ester bonds) are shown as darker spheres. Data file used was kindly provided by Das Sengupta and Siewert-jan Marrink.

chapter, the basic principles of polypeptide binding, partitioning, folding and self assembly in membranes have been discussed.

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