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A Look at Arginine in Membranes

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Abstract Here, we review the current knowledge about the energetics of arginine insertion into the bilayer hydrocarbon core, and we discuss discrepancies between experimental and computational studies of the insertion process. While simulations suggest that it should be very costly to place arginine into the hydrocarbon core, experiments show that arginine is found there. Both types of studies suggest that arginine insertion into the bilayer involves substantial bilayer deformation, with multiple hydrogen bonds between the arginine guanidinium group and lipid polar groups. It is possible that the discrepancies concerning the insertion cost of arginine arise because simulations overestimate the cost associated with bilayer deformation and underestimate the ability of the bilayer to adapt to charged and polar groups. This is an active area of research, and there is no doubt that a consensus view of arginine in membranes will soon emerge.

Keywords Biophysics · Biophysics of ion channels · Structure · Arginine · Bilayer · Hydrophobicity

Introduction

Arginine is often considered to be the most hydrophilic of the 20 natural amino acids. Its side chain contains a large guanidinium moiety that has the capacity for up to six hydrogen bonds (Fig. 1). The resonance-stabilized side chain has a pKa over 12, and thus, arginine is protonated, and positively charged, in essentially all biological environments. However, despite its charged and polar nature, arginine frequently plays a critical functional role within membranes. Membrane-inserted arginines are found in ion channels and other membrane proteins as well as in pore-forming, antimicrobial and cell-penetrating peptides. The function of arginine in membrane proteins and even its presence within the hydrocarbon core of the membrane pose many unanswered questions. In this review we summarize the recent literature and discuss a recent controversy about arginine in membranes.

The arginine controversy is derived from significant discrepancies between experimental and computational studies concerning the energetics of arginine insertion into the bilayer hydrocarbon core. The issues arise because arginine is very hydrophilic and the hydrocarbon core of an unperturbed lipid bilayer membrane is one of the most hydrophobic microenvironments found in nature, with physical–chemical properties that are very similar to a liquid alkane phase (White and Wimley 1999; White et al. 2001). The hydrocarbon core is believed to impart a strict barrier to the permeation of polar or charged solutes through the bilayer. However, the bilayer's hydrocarbon core is positioned between the two chemically heterogeneous interfacial zones, which are composed of some hydrocarbon but mainly lipid polar groups and water. The bilayer interfacial zones thus have many hydrogen-bonding groups that can interact with a membrane-inserted arginine. In this review we propose that these interactions and the ability of the bilayer to deform make it possible for arginine to insert into bilayers, especially when it is surrounded by hydrophobic residues.

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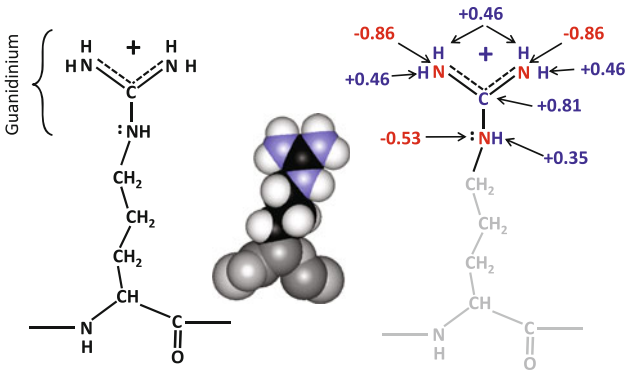

Fig. 1 Physical chemistry of arginine. The side chain of arginine is composed of a hydrophobic propyl moiety and a large, polar, cationic guanidinium group. The resonance-stabilized guanidinium, with a pKa around 12–13, is protonated and cationic under almost all conditions. Nine different atoms in the side chain have significant partial charges, shown on the *right*. The guanidinium group contains five dipolar N–H protons capable of donating hydrogen bonds and one pair of electrons capable of accepting a hydrogen bond. In terms of the potential for interacting with lipid polar groups, it should be noted that hydrogen bond donor moieties are rare in membranes: Phospholipids provide mostly hydrogen bond acceptor groups, in the form of ester bonds and phosphate oxygens

Arginine Becomes Controversial: The Potassium Channel, KvAP

Perhaps the biggest controversy in membrane biophysics in the first decade of the twenty-first century was associated with the structure and function of the voltage-gated potassium channel KvAP (Fig. 2). Voltage-sensitive ion channels control the potassium flow across membranes down the electrochemical gradient and are critical for the propagation of electrical signals in the nervous system. These channels open and close in response to the transmembrane electrochemical potential in a process that involves the motion of the so-called voltage sensor domain. The voltage sensor domain of a classical voltage-gated potassium channel is composed of four hydrophobic segments. One of them (S4) contains at least four arginines, and this arginine-rich helix responds to changes in transmembrane voltage by altering its disposition in the bilayer, thereby opening and closing the channel. In 2003 MacKinnon and colleagues reported, for the first time, the structure of a full-length voltage-gated potassium channel, KvAP, which contradicted previous models of the channel and positioned the four arginines of the S4 segment in direct contact with the hydrocarbon tails of lipids (Jiang et al. 2003a, 2003b). Furthermore, they proposed that the S4 helix moves like a lever arm or a paddle, covering a distance of about 20 Å across the membrane during channel opening and closing. Importantly, these views were based not only on the crystal structure, which was distorted due to the presence of an antibody (which enabled crystallization) attached in close proximity to the S4 helix, but also on electrophysiological assays used to investigate

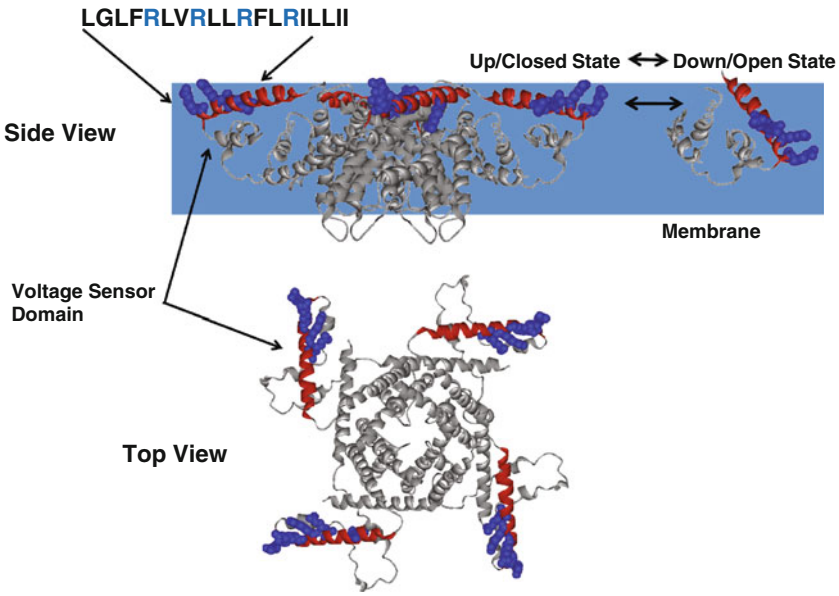

Fig. 2 Structure of the full-length KvAP potassium channel. This membrane-spanning protein has a tetrameric structure with a central channel domain and four voltage sensor domains on the periphery. The voltage sensor domain contains the critical S4 segment, shown as a *red* helix. The S4 segment has four arginines, shown in *blue*, and the arginines are surrounded by a very hydrophobic sequence rich in leucine, isoleucine and phenylalanine residues (*top*). MacKinnon and others (Jiang et al. 2003a, 2003b) have proposed that the S4 segment responds to changes in TM potential by a paddle-like motion that brings the four arginines deep into the membrane in the so-called down state (*upper right*). The proposal that a helix with four arginines can be inserted deep into the membrane generated the controversy we discuss in this review (Color figure online)

the movement of the voltage sensor upon channel opening and closing (Jiang et al. 2003a, b).

Some researchers were highly skeptical of this interpretation. Articles commenting on the KvAP channel structure in 2003 contained comments from scientists who noted that placing an Arg in the hydrophobic environment was "unrealistic" since the hydrocarbon core is a "forbidden zone for charged molecules" (Miller 2003). Based on this classical understanding of arginine in membranes, some researchers concluded that MacKinnon's structural model must be wrong. However, to many, MacKinnon's biochemical and electrophysiological data seemed convincing. Thus, a controversy was born.

The Hydrophilicity of Arginine in Hydrophobicity Scales

How realistic is the idea that Arg is forbidden from entering the bilayer core? The propensity of arginine to be inserted into a membrane depends on its effective hydrophobicity. The hydrophobicity of arginine and the other amino acids has been estimated or measured in many published hydrophobicity scales. Among the commonly used scales, the values for arginine vary considerably. In some early scales (e.g., Wolfenden et al. 1981 and Goldman–Engelman–Steitz [GES, Engelman et al. 1986]) arginine is, by far, the most hydrophilic amino acid, with a free energy of partitioning that is unfavorable by over 13 kcal/mol (relative to Gly). This value essentially precludes the existence of Arg in a membrane. These early scales were based on side-chain partitioning into pure hydrocarbon phases, such as cyclohexane, and are consistent with calculated values for the cost of moving an Arg side chain into a water-free, low dielectric slab of solvent (Parsegian 1969). More recently, however, experimentally derived hydrophobicity scales (Wimley et al. 1996; Wimley and White 1996) have assigned values to Arg that are much less unfavorable. For example, in the context of peptide partitioning into hydrated octanol, the cost of arginine is +0.66 kcal/mol relative to glycine. For partitioning into a bilayer interfacial zone, the value is +0.82 kcal/mol. In these experimentally derived hydrophobicity scales, arginine is actually the least costly of the four charged residues at pH 7, although it is still more unfavorable than the polar, uncharged residues such as Gln, Asn, Ser and Thr (Wimley et al. 1996; Wimley and White 1996).

In a 19-residue sliding window analysis, the Wimley–White whole-residue octanol scale, described above, is an accurate predictor of transmembrane helices in membrane proteins (Jayasinghe et al. 2001; Snider et al. 2009). The difference between the two scales, *octanol-interface*, is also an accurate predictor (Jayasinghe et al. 2001; Snider et al. 2009). Thus, the Wimley–White scales are highly relevant for describing and predicting the real-life propensity of amino acids to be part of transmembrane helices. What do these experimentally derived scales tell us about arginine? In the difference scale (octanol-interface) the value for arginine is actually slightly favorable (by 0.14 kcal/mol) compared to glycine, suggesting that most of the real cost of placing Arg in a membrane has already been paid at the interface. This is not to suggest that Arg is favorable overall because the backbone itself is unfavorable by more than 1 kcal/mol (White and Wimley 1999; Jayasinghe et al. 2001). However, these experimentally measured hydrophobicity scales show that the real cost of arginine partitioning into membranes is far from being as unfavorable as suggested by alkane-partitioning experiments or by some calculations or simulations as discussed below.

The Leucine Equivalent Ratio

To quantitate and compare the cost of partitioning an arginine into a membrane, here we introduce the leucine equivalent ratio, defined as

$$\frac{\Delta G_{\mathrm{Ala}} - \Delta G_X}{\Delta G_{\mathrm{Ala}} - \Delta G_{\mathrm{Leu}}}$$

where the ΔG_X values are free energies for partitioning of amino acid X into a solvent or a bilayer. We chose alanine and leucine as the reference residues because they are common in bilayer-inserted α-helices. The relative cost of arginine partitioning in the context of such a helix can be quantified by comparing the contribution of an Ala → Arg substitution in a hydrophobic helix to that of Ala → Leu. The leucine equivalent ratio is thus the number of Ala → Leu substitutions required to compensate for a single Ala → Arg substitution within a membrane-inserted helix made of mostly alanines. Using the Wimley–White scales, the leucine equivalent ratio for arginine is about 1.7 (Fig. 3). The values for the other charged amino acids are a little higher, up to about 3.0. The widely used Kyte–Dolittle scale, based partially on octanol partitioning, is similar to that of Wimley–White, with leucine equivalent ratios of 3–4 for the charged side chains. The small leucine equivalent ratios for these experimentally derived hydrophobicity scales contrast with the large values calculated from scales based on alkane partitioning. For example, in the Wolfenden and GES scales, the leucine equivalent ratios are 65 and 10, respectively.

Based on the octanol-derived hydrophobicity scales, we conclude that the hydrophobic contribution of *less than two leucine residues* (relative to alanine) is required to overcome the cost of an arginine inserted into a membrane. Furthermore, since it has been shown (Hessa et al. 2005a,

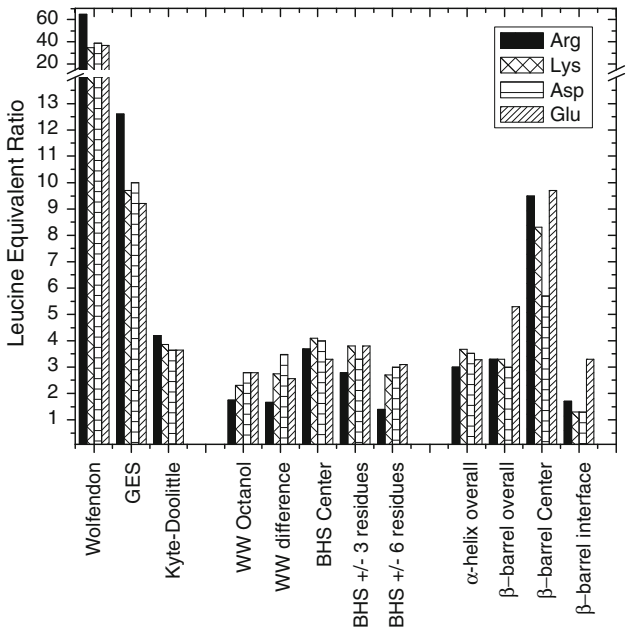

Fig. 3 The hydrophobicity of arginine in membranes can be expressed in terms of leucine equivalent ratios, or the number of Ala → Leu substitutions required to compensate for a single Ala → Arg substitution. The Wolfenden and GES hydrophobicity scales, based in part on nonpolar solvent partitioning, give high ratios, suggesting that it will be very costly to partition arginine into a membrane. On the other hand, the Kyte–Doolittle, the Wimley–White and the biological hydrophobicity scales yield leucine equivalent ratios for arginine that are much smaller. Scales based on the observed abundance of amino acids in membrane proteins of known structure, shown on the *right*, generally agree that the cost of inserting arginine in a membrane is not prohibitive, especially when the arginine is not at the center of the bilayer. These results suggest that it should be possible to insert as many as four arginines in a membrane as long as the surrounding residues are sufficiently hydrophobic

2007; Krishnakumar and London 2007) that a peptide of 19–25 residues comprised of 25% leucines and 75% alanines exists as a transmembrane helix with a favorable partitioning free energy, the calculations suggest that it should be possible to replace as many as four alanines by arginines in such a peptide and still maintain insertion by also changing six to eight other alanines to leucine.

The Hydrophilicity of Arginine in Biological Membranes

Hessa et al. (2005a, 2007) developed an assay system to address the apparent hydrophobicity of the amino acids as detected by the Sec61 translocon machinery. The assay reports the efficiency of translocon-mediated insertion of potential transmembrane (TM) helices into dog pancreas rough microsome membranes. The segment to be tested for membrane insertion is added into the luminal P2 domain of the membrane protein leader peptidase, flanked on both sides by *N*-glycosylation sites. Insertion of the test segment across the membrane results in the glycosylation of only one of the sites, while failure to insert leads to the glycosylation of both sites. SDS-PAGE is used to quantify the fraction of inserted and noninserted segments, yielding apparent equilibrium constants and apparent free energies of insertion. Using this assay, Hessa et al. reported a "biological hydrophobicity scale" for the contribution of the 20 amino acids when placed in the center of a potential 19-residue TM helix. The biological and the Wimley–White octanol scales are generally in good agreement, including the small unfavorable values for arginine and the other charged residues. These measurements support the conclusion that inserting arginine into real membranes is much less costly than previously believed. When hydrophobicity is assessed by the translocon machinery, the leucine equivalent ratio for arginine in the center of a TM helix in a membrane is 4.0. However, Hessa et al. also showed that the apparent free energies of insertion of the charged and polar amino acids depend on the position within the hydrophobic test segment. In particular, it was found that the energetic penalty for Arg insertion into the bilayer is dramatically lower when Arg is moved from the exact middle of the hydrophobic segment. Shifting the position of the Arg by just three residues from the center reduces the leucine equivalent ratio to 2.0, and shifting it by six residues reduces the ratio to just 1. Averaged across the membrane, the overall leucine equivalent ratio in biological membranes is about 2, a value that is not significantly different from the Wimley–White hydrophobicity scale value of 1.7. Again, because a 19-residue leucine/alanine helix with 12 or more alanines inserts stably across a membrane (Hessa et al. 2005a, 2007; Krishnakumar and London 2007), it should be possible for a helix with up to four arginines to insert stably across a bilayer if the remainder of the residues are sufficiently hydrophobic (e.g., mostly leucine).

To address the possibility that the charge in the middle of a TM segment is not actually in the middle of the membrane but shifted toward the interface, Hessa et al. (2005a) performed experiments with pairs of charges that they moved symmetrically away from the center of the test sequence. They found that two charged residues, shifted from the center of the helix, had the expected sum effect. Although the environment that is experienced by the TM helices in the translocon is unknown, these experiments do not support the idea that the charges in the middle of a TM helix are not actually in the middle of the membrane.

Abundance of Arginine in Membrane Protein Structures

Arginine is one of the most uncommon residues in the membrane-inserted portions of integral membrane proteins. The observed abundance values can be converted to apparent free energies (Ulmschneider et al. 2005; Hessa et al. 2007) by taking the ratio of arginine abundance in membranes to its genomic abundance. When the apparent free energies for membrane insertion are compared, arginine is unfavorable but only by about 1.5 kcal/mol. Just as in the biological hydrophobicity scale, the cost of arginine is about four leucine equivalents in the bilayer center, which decreases to as little as 1 a short distance away from the bilayer center. The overall leucine equivalent ratio is about 3 for α-helical membrane proteins, which is very similar to the *overall* value for β-barrel membrane proteins (Wimley 2002; Freeman and Wimley 2010). Interestingly, the abundance of arginine in the bilayer center of β-barrel outer membrane proteins is much lower than for helical membrane proteins. When converted to apparent free energies, the cost of placing Arg in the bilayer center of a β-barrel is about 10.5 leucine equivalents. In the interfacial zone of a β-barrel outer membrane protein, the cost is much lower, giving rise to an overall arginine abundance in β-barrels that is not very different from the abundance in helical membrane proteins (Fig. 3). The higher apparent cost of Arg in the bilayer midplane of a β-barrel outer membrane protein could arise from structural differences in the bacterial outer membrane, where the β-barrels are found, or because a strand of an outer membrane protein only exposes five total hydrophobic residues to the membrane (Wimley 2003) and, thus, cannot have enough extra hydrophobicity to overcome the cost of having a deeply buried arginine as one of the five lipid exposed residues. In any case, the relatively low overall cost of arginine in α-helical and β-barrel membrane proteins shows that arginine can, in fact, be inserted into the membrane in naturally occurring membrane proteins. Taken together, these results make it clear that the bilayer hydrocarbon is actually not a "forbidden zone" for charged molecules. Arginine and other charged residues can, in fact, partition into the core of a lipid bilayer membrane as long as there are enough hydrophobic residues to overcome the cost.

Calculations of Energetics of Arginine Insertion into Membranes

In order to study the partitioning of an Arg residue into a membrane, Dorairaj and Allen (2007) performed a fully atomistic simulation of the insertion process. They calculated the potential of mean force while moving a long poly-Leu helix with a single Arg across a DMPC bilayer. Since the poly-Leu helix is translationally invariant, the potential of mean force yields the free energy of Arg insertion as a function of its depth of penetration into the bilayer. Not surprisingly, the highest potential of mean force was observed for the center of the bilayer. The value, however, was surprisingly large, about 17 kcal/mole, a huge barrier to insertion. The authors argued that the large cost is due to low arginine hydration and a significant strain energy associated with bilayer deformation (Dorairaj and Allen 2007). They further studied how the calculations are affected by the protonation state of the arginine side chain (Li et al. 2008). While they observed insurmountable barriers for both charged and uncharged species, their calculations predicted that arginine will be protonated and charged at neutral pH inside lipid bilayers.

Similarly, MacCallum et al. (2007, 2008) modeled the partitioning of side chains, terminated at the C_β atom, into bilayers. They calculated the potential of mean force using umbrella sampling and observed large variations in partitioning free energies as a function of penetration depth and side-chain chemical composition. For the arginine side chain, this calculation yielded a free energy barrier of about 14 kcal/mole for the center of the bilayer, yielding a leucine equivalent ratio of 11 in the bilayer center. They also observed water penetration into the hydrocarbon core, with water molecules stably surrounding the Arg side chain. Furthermore, they estimated that the charged and neutral forms of arginine will partition similarly in the bilayer center, indicating that there is no driving force for deprotonation.

Experimental Observations of Arginines in Membranes

Despite these simulations suggesting that it should be extremely costly to place arginine into the hydrocarbon core of a membrane, experiments show that arginine can be found there. For instance, one study addressed the effect of arginine on the disposition of the fibroblast growth factor receptor (FGFR3) TM domain in POPC bilayers using neutron diffraction (Fig. 4). The Gly380Arg mutation in this TM domain is known as the cause for the most common form of human dwarfism, achondroplasia. Neutron diffraction and oriented CD experiments revealed that the mutant FGFR3 TM domain is a stable TM helix at equilibrium. Arg380 is buried in the hydrocarbon core of the bilayer and positioned at about 11 Å (six to seven residues) from the bilayer center (Han et al. 2006). On the other hand, the native Gly380 in the wild type is located closer to the bilayer center, at about 6 Å from the bilayer midplane. Thus, the Gly380Arg mutation induces a shift in the TM domain, but in doing so it also brings the native Arg397

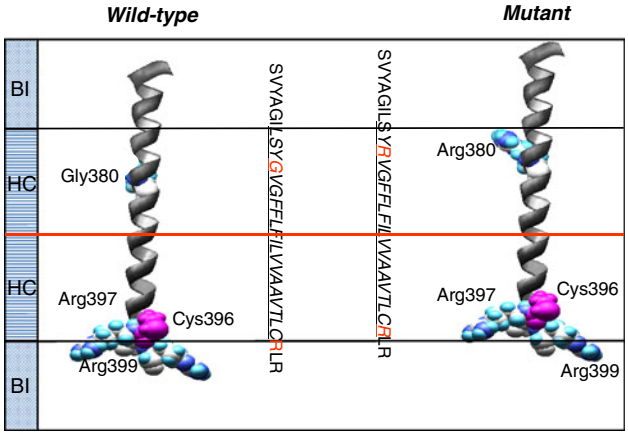

Fig. 4 The disposition of an arginine in a TM helix. A pathogenic mutation in the single-spanning helix of the receptor tyrosine kinase FGFR3 changes a membrane-embedded glycine to an arginine. The Arg-containing helix stably inserts into the membrane, but the positioning of the mutant arginine is shifted out toward the bilayer interface (Han et al. 2006), consistent with the depth dependence of Arg insertion in the biological hydrophobicity scale

and Arg399 to about 13 and 14 Å, respectively, from the bilayer center (Han et al. 2006), such that Arg380, Arg397 and Arg 399 are all positioned in the periphery of the hydrocarbon core. These results are consistent with the view that Arg can insert into the hydrocarbon core but prefers its periphery.

In a second example, the structure of bilayers containing the full-length KvAP voltage sensor was studied using neutron diffraction (Krepkiy et al. 2009). These experiments showed that the voltage sensor incorporates into bilayers with the four domains (S1–S4) oriented in a TM fashion. Thus, the S4 helix is TM in the context of the full-length voltage sensor domain. An important finding of this work was the presence of extensive contacts of the protein with water molecules (Krepkiy et al. 2009), observed in both diffraction and solid-state NMR experiments. However, the overall bilayer perturbation observed in the neutron diffraction experiments was modest, involving a slight bilayer thinning of about 3 Å.

These structural observations of arginine in the hydrocarbon core are consistent with thermodynamic studies of S4 insertion into the endoplasmic reticulum by Hessa et al. (2005b), who measured its apparent free energy of translocon-mediated insertion. They showed that the S4 helix, with four arginines, inserts into the microsomal membrane with an apparent free energy of +0.5 kcal/mol. In other words, the S4 sequence is "poised" around $\Delta G = 0$, as one would expect for a TM domain which needs to respond to changes in TM potential. Contrary to the predictions based on molecular dynamics (MD) simulations, these results demonstrated unequivocally that the four arginines of the S4 segment can indeed be buried in the lipid environment. Thus, there are significant discrepancies between calculations and experiments. We believe that the experimental data are convincing; they utilize a variety of experimental systems and diverse experimental methods. While computational methods have evolved greatly during the past decade, it is still possible that the simulations do not reliably estimate the cost associated with bilayer deformation and the ability of the bilayer to adapt to charged and polar groups.

Insights of Arginine Disposition in Bilayers from MD Simulations

MD simulations have provided insight into arginines inserted into membranes and the membrane's response to insertion. In one example, Freites et al. (2005) simulated the S4 helix inserted across membranes. They showed that insertion of the four arginines into the bilayer as part of the hydrophobic S4 peptide leads to a local "extreme distortion of the bilayer." This distortion is manifested in high local water penetration, a dramatic decrease in the thickness of the bilayer hydrocarbon core to about 10 Å and unusual conformations of lipids that interact directly with arginines via hydrogen bonds. The simulations further showed that none of the four arginines was ever in direct contact with lipid hydrocarbon and revealed a network of hydrogen bonds around the guanidinium groups. Each guanidinium group participated in three hydrogen bonds throughout the simulation trajectory, including hydrogen bonds to lipid phosphate groups, other lipid polar groups and water. It is worth noting that phospholipid polar groups contain mostly hydrogen bond acceptor groups, in the form of ester bonds and phosphate oxygens, while the guanidinium moiety contains mostly hydrogen bond donor groups in the form of NH hydrogens. These persistent hydrogen bonds and the ability of the bilayer to deform lead to the penetration of water and lipid phosphate groups into the hydrocarbon core, along with the arginines. Thus, the hydrogen-bonding capabilities of Arg and the ability of the bilayer to deform locally appear to be critical for the insertion and the function of the S4 helix in bilayers.

MD simulations of the full-length voltage sensor domain further showed that the protein perturbs the bilayer structure and that the perturbations are restricted to the lipids in very close proximity (Krepkiy et al. 2009), which are significantly distorted to accommodate the arginine residues in the membrane. Furthermore, almost 50 water molecules were found to be associated with the membrane-embedded protein. These bound water molecules were proposed to play an important role in the function of the voltage sensor domain (Krepkiy et al. 2009).

Arginine in Translocating and Antimicrobial Peptides

Arginine is also surprisingly abundant in membrane-active antimicrobial and membrane translocating peptides, with some examples having arginine contents as high as one-third (White et al. 1995; Yount and Yeaman 2004; Jiang et al. 2008). Recent work has shown that these classes of peptides probably function by a mechanism that relies on their "interfacial activity" (Rathinakumar and Wimley 2008; Rathinakumar et al. 2009) or the propensity to partition into the bilayer interfacial region and drive lipid rearrangements that break down the vertical segregation between lipid polar and nonpolar groups. We propose that this mechanism relies on (1) the ability of arginine to form multiple electrostatic and hydrogen-bonding interactions with the lipid headgroups and water and (2) the ability of the bilayer to deform in response to these interactions. Thus, arginine-containing peptides, driven deep into the bilayer by the hydrophobic contribution of nearby moieties, bring lipid polar groups and water with them into the core of the membrane. This disrupts the strict segregation of the polar and nonpolar groups of the membrane and allows for the movement of the lipid polar groups, water and the peptide itself across the hydrocarbon core. These are exactly the kind of interactions observed in the simulation of the S4 helix and the whole voltage sensor domain in membranes (Freites et al. 2005, 2006). Thus, we propose that it is the hydrogen bonding between the guanidinium group and the lipid headgroup moieties and water that allows arginine-containing, hydrophobic sequences to penetrate deep into the membrane. Unlike the S4 helix, pore-forming or translocating peptides cause bilayer destabilization or permeabilization. We hope that future experiments and simulations will reveal why some Arg-containing peptides are membrane-translocating, some are membrane-permeabilizing and others have little or no effect on membrane structure.

Conclusion

Since the publication of the KvAP channel structure, the structural and thermodynamic aspects of arginine insertion into membranes have been the subject of intensive research and discussion. Our understanding of the subject has evolved. It is clear that arginine can be positioned in the bilayer at the depth of the hydrocarbon core. However, arginine is not in direct contact with hydrocarbon groups as it maintains strong interactions with water molecules and lipid headgroups in a highly perturbed lipid bilayer environment. Hydrogen bonding between the guanidinium group, polar lipid moieties and water molecules is critical. However, MD simulations still show a very high cost of arginine insertion, and thus, a significant discrepancy remains. We look forward to new experimental and computational results, further insights into the response of the bilayer to inserted arginines and a consensus view of the energetics of arginine insertion into membranes.

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