

Hydrophobicity scales: a thermodynamic looking glass into lipid–protein interactions

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The partitioning of amino acid sidechains into the membrane is a key aspect of membrane protein folding. However, lipid bilayers exhibit rapidly changing physicochemical properties over their nanometer-scale thickness, which complicates understanding the thermodynamics and microscopic details of membrane partitioning. Recent data from diverse approaches, including protein insertion by the Sec translocon, folding of a small beta-barrel membrane protein and computer simulations of the exact distribution of a variety of small molecules and peptides, have joined older hydrophobicity scales for membrane protein prediction. We examine the correlations among the scales and find that they are remarkably correlated even though there are large differences in magnitude. We discuss the implications of these scales for understanding membrane protein structure and function.

Complexity of protein–lipid interactions

Membrane proteins exist in a unique environment where over a length scale of 4 nm, the length of a single transmembrane helix, the physicochemical properties change from nearly bulk water through a concentrated electrolyte solution, an ordered hydrophobic matrix, a disordered hydrophobic solvent and back (Figure 1). This rapidly changing environment confounds understanding the energetics of membrane protein folding and insertion, as well as the mechanism of action of peptides and proteins that interact with membranes in a variety of biological processes.

The earliest membrane protein prediction methods were based on the partition coefficients for small molecule mimics of amino acid sidechains between solvents of different polarity [1]. Such scales can be understood purely in terms of the partitioning between two relatively simple phases. In this review, we discuss recent work that questions whether such simplified systems are appropriate for understanding the interactions of proteins with lipids within biological membranes.

The thermodynamic and microscopic details of lipid–protein interactions have become acutely important in a number of key biological problems. The first crystal structure of a voltage-gated potassium channel, KvAP [2], gave

rise to vigorous discussions about the energetics of the interactions between arginines and lipids, as the structure suggested a gating mechanism in which charged arginines were exposed to the hydrophobic bilayer interior.

The action of antimicrobial peptides and cell-penetrating peptides has also raised questions about lipid–protein interactions. Anti-microbial peptides often have specific amino acid sequences enriched in cationic and aromatic residues, whereas cell-penetrating peptides are rich in cationic residues [3].

In a third major development, the astonishing success in crystallizing and determining the structure of the Sec translocon system, the fundamental machinery that inserts membrane proteins into the membrane as they are synthesized by the ribosome, has raised questions about the thermodynamics of membrane insertion [4,5].

In this review we compare hydrophobicity scales based on experiments with increasing biological complexity. The simplest scale is based on partitioning of small molecules between bulk solvents. The most complex scale is based on *in vitro* experiments on the insertion of a transmembrane helix by the Sec translocon. We use computer simulation results to interpret some of the experimental data and discuss the implications of the different scales for membrane protein structure prediction and biophysical understanding of lipid–protein interactions.

Membrane structure

The membrane bilayer is highly heterogeneous in the normal direction, with large gradients in density and polarity on a nanometer length scale (Figure 1) [6]. We divide the bilayer into four regions based on physicochemical properties [7]. Although the four-region model is a useful membrane roadmap, there are three important caveats. First, biological membranes contain a diverse mixture of lipids with different properties that is more complex than a single-component lipid bilayer. Second, biological membranes contain a large fraction of membrane proteins, sometimes as high as 25% by area [8]. Indeed, the presence of protein may complicate a thermodynamic description of lipid–protein interactions considerably. Finally, it is now well established, based primarily on computer simulations, that the primary response of a bilayer to the insertion of polar and, in particular, charged

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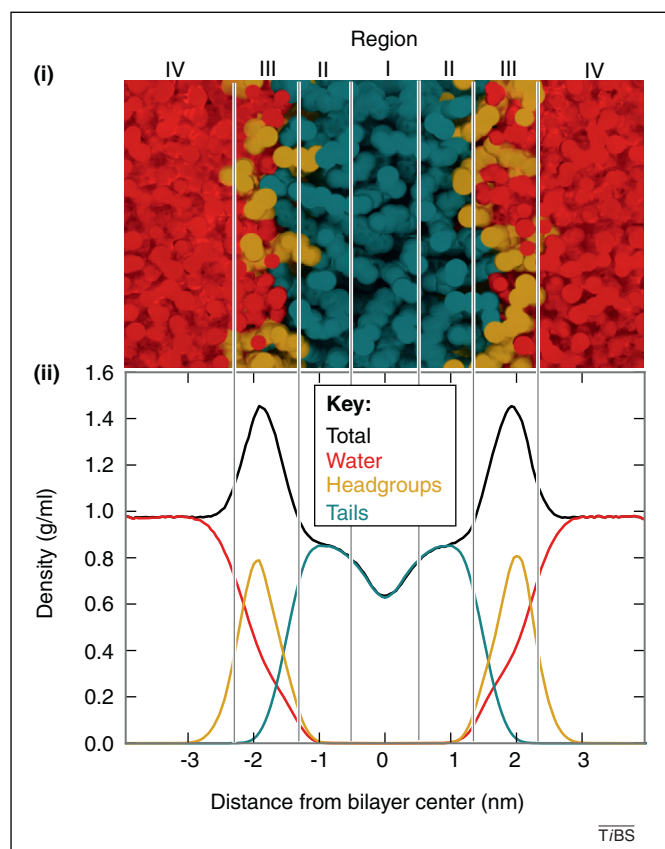


Figure 1. Lipid bilayers contain large variations in density and polarity on a nanometer scale. (i) Snapshot of a DOPC bilayer. (ii) Partial density profile of a pure DOPC bilayer. The system is divided into four regions with different physicochemical properties [7]. Region I, the center of the bilayer, is hydrophobic and significantly disordered with properties similar to decane. In Region II, the lipid tails are more ordered and have a higher density, similar to a soft polymer. Region III contains a diverse mixture of functional groups including the carbonyl and glycerol groups of the lipid tails, most of the head group density and water. Region IV is defined by water that is perturbed by the lipid bilayer and can be quite deep.

groups is to distort the lipid–water interface so that effectively the four regions become locally deformed [9–12]. Despite these caveats, the four-region model is a useful conceptual starting point for thinking about membrane structure.

Importance of hydrophobicity scales

Structural biology of membrane proteins remains a challenging area of research. Only ~290 unique membrane protein structures are known (<http://blanco.biomol.uci.edu/mpstruc>, accessed June 23, 2011), in stark contrast with the total of ~68,000 structures in the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/statistics/holdings.do>, accessed June 23, 2011). This is particularly sobering when considering that as many as 20–30% of all proteins are membrane proteins [13,14] and the majority of currently approved drugs interact directly with membrane proteins [15–17]. Membrane proteins do appear to follow simpler structural principles than water-soluble proteins; they are primarily helical, with the major exception of beta-barrel proteins found in the outer membranes of bacteria and mitochondria. In addition, membrane proteins are confined in space by the lipid environment. These restrictions offer hope for computational prediction of membrane protein structures, at least at a low level of resolution. Indeed,

one of the main practical uses of hydrophobicity scales is to recognize transmembrane helices in amino acid sequences. This provides a starting point for identifying the topology of membrane proteins, a major step in the design of experiments and in the identification of regions of structural or functional importance.

In addition to being a computational tool to recognize transmembrane sequences, the hydrophobicity scales outlined in this review also provide physical insight into lipid–protein interactions. One reason for the slow progress in membrane protein structural biology is that the physical driving forces for membrane protein folding and stability remain poorly understood [18,19]. Although simplified models based on helix insertion and folding are widely used [20–22], we still lack understanding of the detailed energetic balance between water, lipids, and proteins in various states of folding, unfolding and aggregation. Recent studies on helix insertion into the membrane by the Sec machinery have put these conceptual models in a more directly biological context and suggest that the decision to insert a helix into the membrane is based on simple physical chemistry principles [5,23], but the second stage of this process, folding into a larger membrane protein, is more complicated. Further, not all membrane proteins require the Sec machinery.

It has become increasingly clear that lipids play crucial roles in both membrane protein folding and function [24]. Although there are a few high-resolution membrane protein structures that also resolve some lipid or, in some cases, surfactant density and detailed structural data on lipid–protein interactions remain rare [25]. Experimental hydrophobicity scales reveal the thermodynamics of interactions between sidechains and lipids and have the potential to provide insight into more complex phenomena, such as non-additivity and cooperative effects.

A focus on basic thermodynamics also makes these scales useful in understanding a variety of other processes that involve partitioning of a diverse range of chemical groups, both by themselves and in complex molecules. Examples include antimicrobial peptides, peptides and other molecules designed to transport cargo across the membrane, and hydrophobic cationic drugs that permeate passively through a membrane. Finally, the simpler systems provide important data for the development of simulation methods and parameters (see [26]).

Physics-based hydrophobicity scales

In this review, we consider five approaches for deriving hydrophobicity scales from molecular interactions (Figure 2). Three of these are based on experiments that measure thermodynamic equilibrium through partitioning or folding/unfolding protein equilibria, one is based on computer simulations and one is based on the biological Sec translocase system.

Radzicka: The Radzicka–Wolfenden small molecule partitioning scale

Radzicka and Wolfenden developed an early hydrophobicity scale based on partitioning of small molecule analogs of amino acid sidechains between water and cyclohexane (Figure 2, Radzicka) [27,28]. Although initially developed

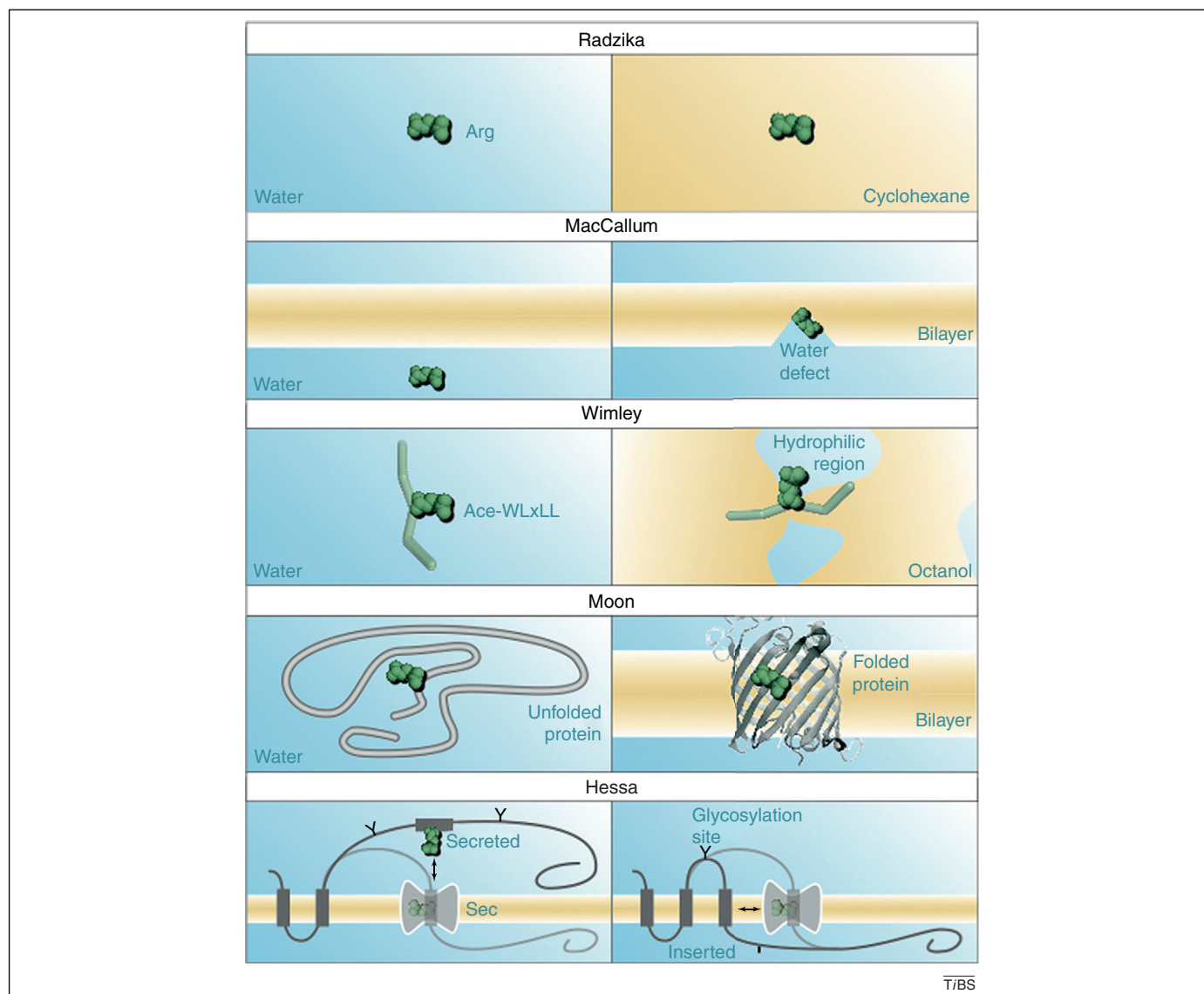


Figure 2. Summary of systems and environments in the different hydrophobicity scales addressed in this work. Each panel shows an overview of the system studied by the experiment and an indication of the environment encountered by an arginine residue.

to understand the folding of globular proteins, this scale is relevant for membrane partitioning because the center of the membrane has physicochemical properties similar to those of bulk hydrocarbon.

The sidechain analogs are added to a biphasic system of water and cyclohexane. After equilibration, the concentration is measured in each phase and the free energy of transfer is determined from the ratio of concentrations. In these experiments, the local microenvironment of each residue is well defined; it is surrounded by water or by cyclohexane.

Although conceptually simple, there are two caveats for applying this scale to membrane partitioning: (1) lipid bilayers (Figure 1) do not resemble isotropic solvents; (2) sidechains by themselves ignore important aspects of protein structure, most notably the backbone.

MacCallum: The MacCallum et al. molecular dynamics potential of mean force scale

MacCallum *et al.* used molecular dynamics simulations to calculate the distribution (Figure 2, MacCallum) of the

Radzicka-Wolfenden sidechain analogs in a 1,2-dioleoyl-sn-glycero-3-phosphocholine bilayer (DOPC, a lipid with two mono-unsaturated tails with 18 carbons each). The free energy of partitioning between water and any region of the membrane can be calculated from these distributions [11,29]. The results include the full response of the lipid bilayer and water but, as above, are limited to sidechain analogs. They show that glutamate and aspartate quickly become neutral, whereas lysine maintains its charge somewhat further into the bilayer and arginine does not become deprotonated [29]. The local environment of each residue is complex and depends on the chemical nature of the sidechain and the location in the bilayer; however, because these are computer simulations, the local environment is known in atomic detail. One of the most striking features is the importance of the formation of water defects, local deformations in the membrane that allow water to penetrate into the bilayer core and maintain hydration of charged and polar groups, in partitioning of polar and charged molecules into the membrane

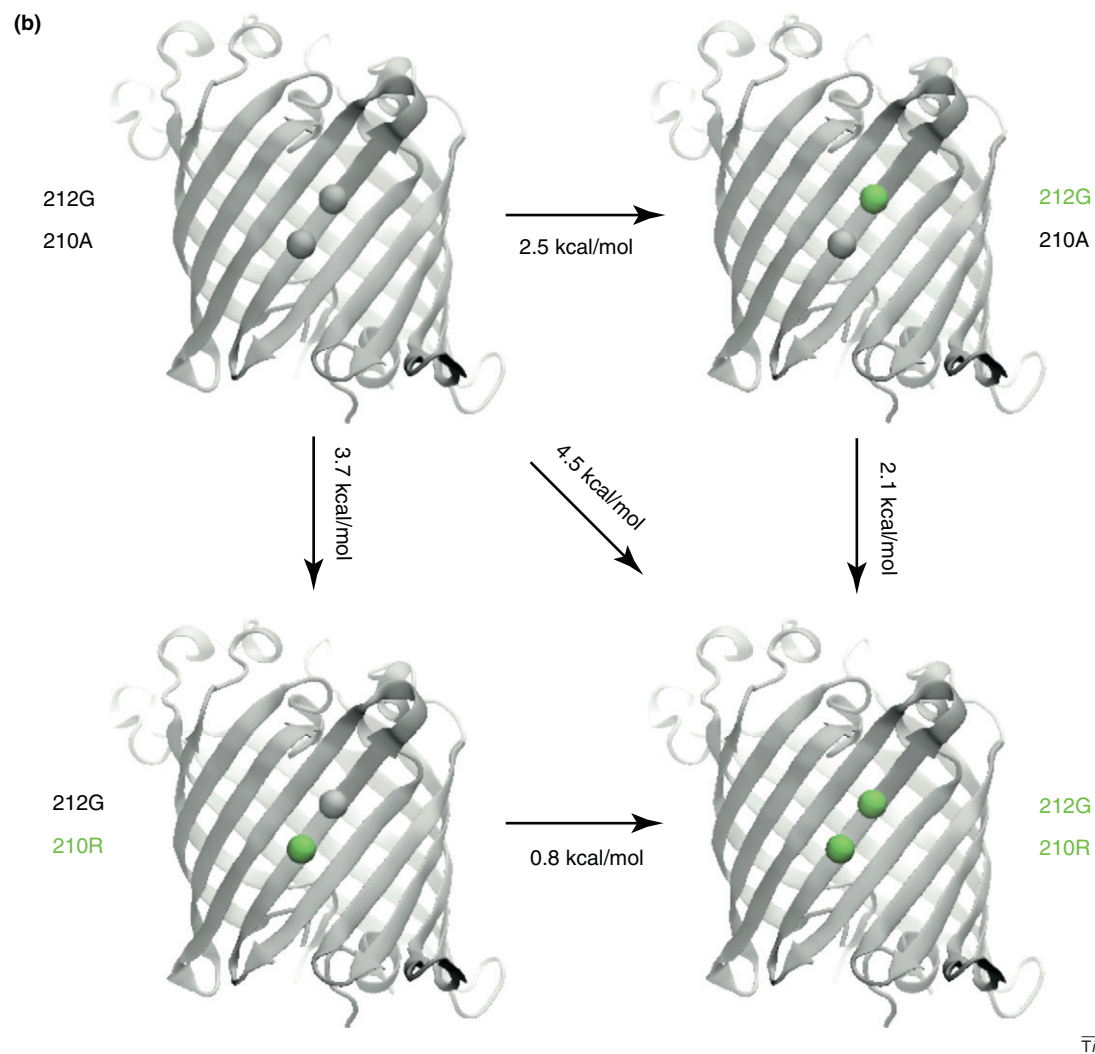
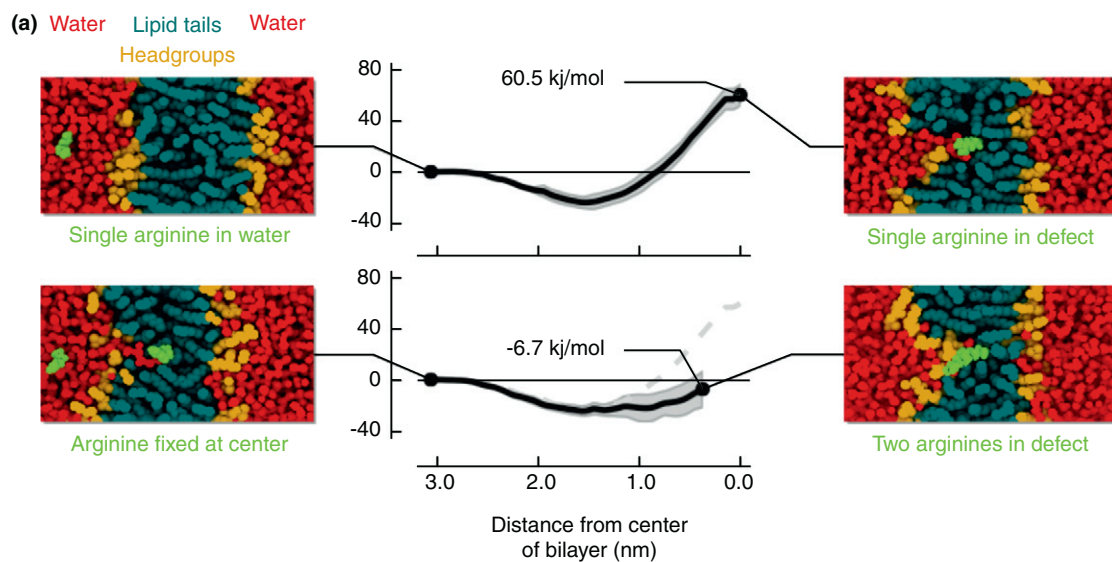


Figure 3. Arginine partitioning into lipid bilayers is non-additive. (a) Calculations show that arginine causes a water defect in the membrane. Adding a second arginine to an existing defect causes almost no increase in free energy. Adapted with permission from [47]. (b) Experimental observation of non-additivity of arginine partitioning. This panel summarizes five different experimental observations. Adapted with permission from [35].

(Figure 3a). Water defects have now been shown in numerous simulations using different protocols and parameter sets [10,30].

Wimley: The Wimley–White pentapeptide-based hydrophobicity scales

The importance of the thermodynamics of lipid–protein interactions was recognized early on by White and co-workers, who developed a peptide-based system to derive a thermodynamic scale for interactions between sidechains and lipids [31]. For each of the pentapeptides Ace-WLxLL, where x is any of the 20 naturally occurring amino acids, they measured the partitioning between water and 1-octanol [32] and, by equilibrium dialysis and reverse-phase HPLC, the partitioning between water and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, a lipid with one saturated tail with 16 carbons and one mono-unsaturated tail with 18 carbons) vesicles [31].

The hydrophobic residues in the pentapeptide host ensure interactions of the peptide with the lipid–water interface, but the unsatisfied hydrogen bonding requirements of the backbone and polar ends of the peptide ensure that the peptide cannot penetrate deeply into the membrane. Thus, the POPC–water scale specifically measures interfacial partitioning. Because this scale is explicitly interfacial, whereas the other scales focus on the bilayer core, we will not discuss it further.

The octanol environment is heterogeneous (Figure 2, Wimley), with local microstructures caused by clustering of hydroxyl groups and the small amount of water present in octanol in equilibrium with water [33,34]. By extending the peptide length, the thermodynamic contribution of the backbone peptide bond was also determined [32]. This 2 kcal/mol per peptide bond contribution compels the formation of secondary structure inside the membrane in order to avoid unsatisfied hydrogen bonds.

Compared to the small molecule scale, the peptide-based scales are a major step towards more realistic systems, but they emphasize the interactions of sidechains with either the water–lipid interface or a heterogeneous octanol environment that can offer a hydrophobic environment to a leucine sidechain, but a more hydrophilic environment to an arginine sidechain. A detailed understanding of these experiments will require microscopic insight.

Moon: The Moon–Fleming OmpLA folding/refolding scale

Recently, Moon and Fleming developed a scale based on the reversible *in vitro* equilibrium between the water-soluble unfolded state and membrane-inserted folded state of outer membrane phospholipase A (OmpLA) [35]. This is a powerful approach that relies on measuring a true thermodynamic equilibrium. By making mutations in the well defined structure of OmpLA, the equilibrium between the folded, membrane-inserted state and the unfolded state in solution can be shifted (Figure 2, Moon) and measured by fluorescence spectrometry, and a full scale for all amino acids can be made. For all mutations, the enzymatic activity of OmpLA was measured to verify correct folding and insertion. The large beta-barrel guarantees that the protein is inserted into the membrane and provides control

over the location of the mutated sidechain. The experiment compares a well-defined folded membrane state with an unfolded state in solution, in which the microenvironment of the mutated sidechain is not known exactly. The 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC, a lipid with two saturated tails with 12 carbons) bilayers used in the experiment are relatively thin and unstable, which may further complicate understanding the experiment.

Hessa: The Hessa *et al.* Sec translocon hydrophobicity scale

Hessa *et al.* developed a hydrophobicity scale based on a previously developed membrane protein insertion assay using the small membrane protein leader peptidase (Figure 2, Hessa). Leader peptidase normally has two transmembrane helices that insert into the membrane in a well-defined orientation with both the N-terminus and the C-terminus in the lumen of the endoplasmic reticulum. Hessa *et al.* engineered two glycosylation sites and a 19-residue insertion, the H segment, which, depending on its hydrophobicity, may be inserted into the membrane as a transmembrane helix. The insertion state of the H segment can be determined by assaying the two glycosylation sites. An apparent equilibrium between inserted and non-inserted H segments can be achieved and quantified by modulating the sequence [36,37].

In this experiment, insertion involves the Sec translocon, the usual cellular machinery that either inserts or secretes a given segment as it comes off the ribosome. The location of the guest residue in the H segment can be varied to determine the effect of location along a helix on partitioning, and multiple guest residues can be included to study additivity and cooperativity [37]. On the basis of these data, Hessa *et al.* developed a transmembrane prediction method that relies on a linear combination of single residue results to predict whether a helix will insert into the membrane [37].

This method has also been applied to test whether a particular sequence from a voltage-gated potassium channel would insert into the membrane, despite having multiple arginine and other polar residues [38].

This experiment measures the apparent free energy difference between the inserted and secreted states of the H segment. In each case, the local microenvironment is not known [39]. For example, residues in the H segment might be interacting with other parts of the protein rather than interacting with lipids or water. As discussed below, insertion by the translocon might also be a non-equilibrium process.

Comparing the scales

Although the different scales measure different things, all but the POPC–water peptide scale (not shown) correlate well with each other (Figure 4). There is no reason *a priori* to expect correlation between the scales, especially given the different systems, methods and assumptions involved in each experiment. The Radzika–Wolfenden and MacCallum scales correlate very well and give almost the same absolute free energy differences. The other three scales involve more complex environments for the ‘guest’ residues. The Wimley–White scale measures interactions in the heterogeneous

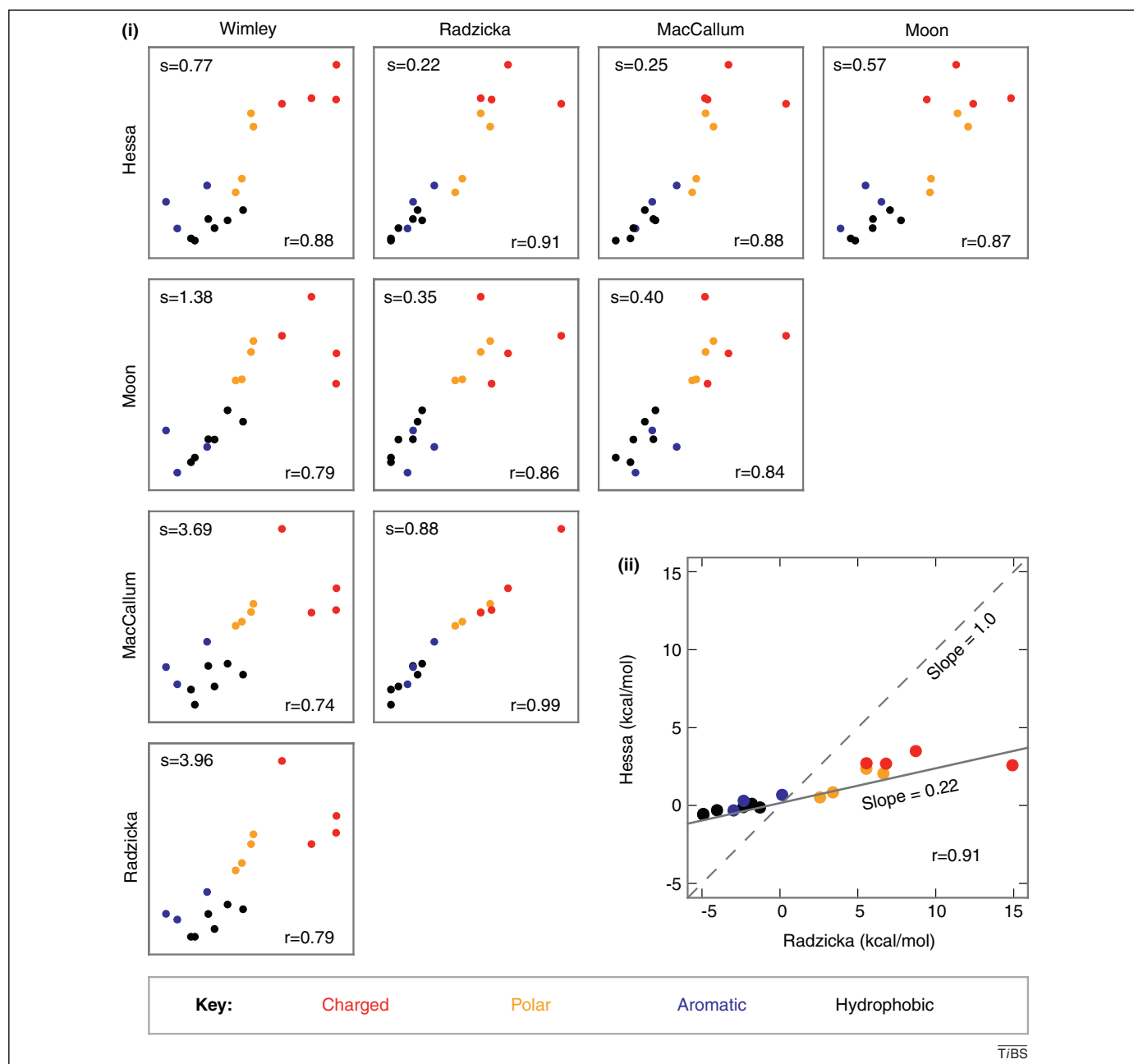


Figure 4. The hydrophobicity scales are correlated but differ in overall magnitude. **(i)** Correlation between the normalized scales, in order to emphasize the correlation (r) among the scales; all scales are normalized so that the values range from -1.0 to 1.0 . The scales differ in overall magnitude, as indicated by the slope (s). **(ii)** Hessa and Radzicka scales are correlated, but differ in overall magnitude scale factor. This panel plots the energies on an absolute scale to emphasize the difference in overall magnitude. Residues are colored by type. His, Pro, Gly were not present in all scales and are not shown. The values for Glu and Asp in Moon might represent a partially neutral state as the experiments were done at pH 3.8.

water-octanol environment, whereas the Moon–Fleming and Hessa *et al.* scales measure properties directly related to membrane protein insertion and stability. However, these scales involve the complex environments of the Sec translocase and the unfolded protein as reference states.

The absolute magnitudes of the Wimley–Hessa–Moon scales and the MacCallum–Radzicka scales differ by a significant amount (shown as the slope, s , in Figure 4). Interestingly, a scale based on the yeast Sec61 translocase correlates well with the original Sec translocase scale, but the absolute difference between the most hydrophobic and most hydrophilic residues is a factor of ~ 2 lower for the yeast scale [40].

How can these differences be reconciled? Where does the large difference in absolute scale come from? It is also of interest to consider how the measured values for single residues translate to multiple residues, which is a functionally more important case.

The two simplest scales, Radzicka and MacCallum, are unambiguous and easily understood. However, they lack several essential details: both lack the polypeptide backbone entirely and Radzicka considers only a simple hydrophobic solvent that differs from a lipid bilayer in many ways.

The remaining scales, Wimley, Moon and Hessa, are based on more realistic systems involving partitioning of

peptides or proteins between complex phases. These experiments are, however, more difficult to interpret because the local microenvironment of the sidechain is unknown. It is possible that different sidechains have different local environments, which greatly complicates the interpretation of these experiments [39].

Possible non-equilibrium effects

Because the Hessa *et al.* scale is based on a biological system, it has been suggested that it does not measure a true thermodynamic equilibrium [41,42]. However, the predicted distribution of amino acids in transmembrane helices by the Hessa scale agrees well with the distribution obtained from a database of known membrane protein structures [43]. Moreover, the Hessa scale is highly correlated with the other scales (Figure 4).

If the translocon decides, based on molecular interactions, as appears to be the case, whether a helix is inserted into the membrane rather than secreted, then helix insertion cannot be a true thermodynamic equilibrium and some measure of kinetic stability is involved in membrane topology and structure. If the translocon only catalyzes insertion, then a true thermodynamic equilibrium would be established between a membrane-inserted and a membrane-secreted form. In both processes, the translocon itself is not a thermodynamic reference state for membrane protein insertion.

It seems likely, however, that the Hessa experiments do not measure an overall equilibrium between soluble and membrane-inserted forms but rather describe a pseudo-equilibrium at one step: transfer of a helix in the complex translocon system to the membrane, in the presence of whatever other helices and lipids might already be in the membrane. Computer simulations of a helix containing an arginine sidechain from the translocon to lipid give much closer agreement with the experimentally measured value than simulations of insertion of an arginine without the Sec translocon [44]. However, if the translocon can influence whether a given segment is inserted into the membrane, then the overall process must be non-equilibrium as the helix bound to Sec is not one of the end-states.

Origin of the large differences in magnitude

In general, the Hessa scale is 'compressed' relative to the more biophysical scales (Figure 4), by a factor of 4 compared to the sidechain scales of Radzicka and MacCallum, while the difference with the Wimley octanol scale is much less (factor of 0.8). The newest scale, the Moon scale, is somewhat intermediate.

Whatever mechanism accounts for the differences between scales, it must explain the fact that the different scales are highly correlated. The differences cannot arise from simple constant shifts in free energy, as would be expected if the differences were due to overall differences in the hydrophobicity of the phases used in each experiment. Rather, the scales differ by a multiplicative factor: if the polar residues have a higher free energy in one scale relative to another, then the non-polar residues will have a more favorable free energy of transfer so that the ratio of free energies is similar.

For the charged residues, lower values compared to the small molecule scale can be explained by a more hydrophilic environment inside the membrane than in the small molecule scales, and/or a less hydrophilic environment in the soluble phase. This is consistent with calculations on coarse-grained models of the translocon [41] as well as with atomistic simulations of the translocon [44] and model helices [30]. Recent molecular dynamics simulations of OmpLA with an arginine guest residue [45] show that the arginine sidechain points towards the water face and is immediately engulfed in a water defect, at a cost that corresponds more closely to the much lower value for sidechain partitioning closer to the interface than to the membrane center.

However, to explain the compression of the full scale we also need to explain the lower absolute values for the hydrophobic and small polar molecules. The free energy of transfer from the translocon to lipid for a leucine sidechain is also less than expected for a small molecule, owing to the less unfavorable environment inside the translocon compared to bulk water [44]. Recent experiments using nonproteinogenic aliphatic and aromatic sidechains show that the insertion of non-polar residues depends on the accessible non-polar surface area, although with a surface tension of 6–10 cal/Å² rather than 23 cal/Å² as would be expected for bulk hydrocarbons [46].

Taking all of the simulations together, the results suggest that the biological scales are compressed compared to the small molecule scales because insertion involves transfer from an environment that is less polar than pure water to an environment that is less hydrophobic than pure hydrocarbon. This is also consistent with the microenvironment that sidechains encounter in octanol, which might be a better mimic of a protein-rich membrane environment than the hydrophobic environment most small molecules (except the arginine and lysine analogs) encounter in the membrane or bulk solvent.

The change in scale factor between the Moon, Wimley, Hessa and the Hessa yeast-Sec scale thus reflects different environments of either the inserted or the second state (inside the translocon, in the unfolded protein, or in water), or both.

Additivity versus non-additivity

The above-mentioned scales are based primarily on single residues, although in principle they can address the thermodynamics of partitioning of multiple residues.

MacCallum *et al.* explicitly considered the partitioning of two or three arginines restrained to be at specific distances from each other [47]. Although the cost of moving one arginine sidechain into the membrane is substantial, determined primarily by the cost of making a water defect in the bilayer, a second or even third arginine is almost free in terms of free energy because the water defect is already present (Figure 3a). Free energy calculations on more complex combinations, as well as full helices, are possible, but it is currently very costly in terms of computer resources to obtain accurate and systematic results [48,49].

Moon and Fleming considered experimentally what happens if two arginines are present on the outside of OmpLA (Figure 3b) [35]. Although the degree of cooperativity is not

as high as in the simulations of the simple sidechains, the cost of moving two arginines into the membrane is less than twice the cost of moving one arginine into the membrane. Because the cost of water defect formation extending to the center of thinner membranes is less than in thicker membranes [50], the cooperativity can be expected to be lower as well. This system shows considerable promise for further thermodynamic insights into membrane protein folding and stability.

Hessa *et al.* have also studied pairs of residues as well as the effect of their location. The location along the helix had a strong effect on charged and strongly polar residues, perhaps consistent with the formation of water defects. The helix-breaker proline also displayed strong position dependence. Aside from proline, symmetric pairs of residues introduced into the H segment showed no cooperative effect [37].

Implications

What can we learn from the different hydrophobicity scales? They have implications in a number of areas.

For partitioning

At perhaps the most basic level, scales like the Hessa, Moon and MacCallum scales, and related work on peptide–lipid interactions will change the textbook view of interactions between polar or charged molecules and membranes. These scales make it clear that the cell membrane interior is complex and heterogeneous. Further, the lipid bilayer is dynamic and deformable, resulting in lower free energies than previously expected.

Going beyond single-residue scales, one of the most interesting results of the Moon scale and recent simulations of multiple arginines is the demonstration that multiple arginines, and by extension other charged or polar groups, show cooperative behavior as they are placed inside the membrane. In general, this behavior is consistent with a model in which a leading charge creates a water defect, after which additional charges are thermodynamically not very costly [10]. This could be relevant for the mechanism of antimicrobial [51] and cell-penetrating peptides [52] as well as for membrane proteins that rely on water penetration into the bilayer for their function.

For membrane protein folding/insertion

The details of the physical basis of membrane protein folding remain elusive. The Sec translocon plays a key role in biology, but is not necessary for all membrane protein folding. It is unclear whether Sec plays an active role in deciding the fate of a given segment or if it is simply a catalyst. The processes involved in recognition and selection of amino acid sequences for either insertion into the membrane or secretion outside the membrane and the structural role of Sec has been reviewed recently [4,5]. The hydrophobicity scales described here help to establish the basic thermodynamics of this process, which will have to be taken into account in developing a detailed mechanism of membrane protein insertion. Computer simulations should be a helpful tool in this endeavor [53]. The Hessa *et al.* scale is obviously directly relevant for the mechanism of Sec, but further progress in structural

biology will be crucial for understanding the details of membrane protein insertion [54]. A key result of the experiments described in this review is the recognition that the relevant thermodynamic process is not the partitioning of a transmembrane helix from pure water into pure hydrocarbon, but that in both cases the environment is more complex.

For prediction

We used the Membrane Protein Explorer [55] software package to predict the transmembrane helices of the ATP-binding cassette (ABC) transporter BtuCD, which has 10 transmembrane helices in each of two identical subunits (Figure 5). Although the scales give different profiles, almost identical predictions arise following normalization. This is encouraging, as the scales give relatively consistent

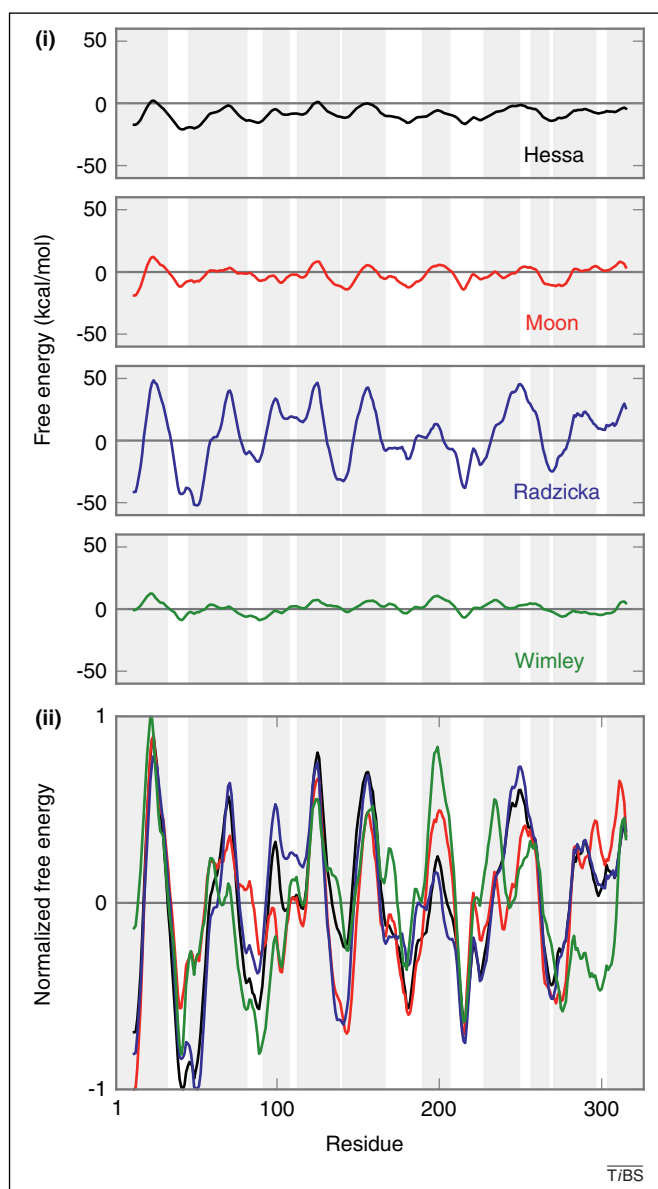


Figure 5. Hydropathy analysis of the protein BtuCD predicts similar transmembrane segments using different scales. (i) Free energy for a 19-residue stretch centered on each residue as calculated by Membrane Protein Explorer [55]. (ii) Normalized values of the four scales. Each scale is normalized to lie within the range -1.0 to 1.0 . Gray shading indicates experimentally observed transmembrane segments. MacCallum and Radzicka scale results are similar and are omitted for clarity.

predictions despite otherwise large differences, but it shows also that it is difficult to tell which scale is most relevant based on experimentally observed transmembrane segments. It is noteworthy that two prediction methods based on the Hessa *et al.* results [37], which have an ~80% accuracy rate [49], are comparable to the best machine-learning methods that draw upon hundreds of parameters.

Hydropathy analysis is based on an explicit assumption of additivity, but the results of both Moon and MacCallum scales bring this assumption into question. So far, non-additivity has been demonstrated for only arginine residues, which may be the most extreme case. Further study is required to examine if similar non-additivity effects are important for other residues, and if taking non-additivity into account might improve the prediction of marginally hydrophobic helices or helices that might interact with additional helices [56].

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