

A sliding selectivity scale for lipid binding to membrane proteins

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Abstract

Biological membranes form barriers that are essential for cellular integrity and compartmentalisation. Proteins in the membrane have co-evolved with their hydrophobic lipid environment which serves as a solvent for proteins with very diverse requirements. As a result, their interactions range from non-selective to highly discriminating. Mass spectrometry enables us to monitor how lipids interact with membrane proteins and assess their effects on structure and dynamics. Recent studies illustrate the ability to differentiate specific lipid binding, preferential interactions with lipid subsets, and nonselective annular contacts. Here, we consider the biological implications of different lipid-binding scenarios and propose that binding occurs on a sliding selectivity scale, in line with the view of biological membranes as facilitators of dynamic protein and lipid organization.

Lipids as solvent for membrane proteins

The lipid composition of biological membranes is highly complex. Lipids can possess different head-groups, alkyl chain lengths, degrees of saturation, and further chemical modifications, all of which contribute to their individual physical properties. Membranes themselves contain a large number of structurally distinct lipids whose ratios may change in response to environmental conditions. Their compositions can differ dramatically between organisms, as exemplified by the dissimilar membrane structures of extremophiles and bacteria. Given this variability, it is almost surprising that integral membrane proteins, which are adapted to a specific host membrane, can have close homologues in all domains of life. This has led to the suggestion that lipids primarily function as hydrophobic solvents for membrane proteins [1,2], much like soluble proteins that commonly tolerate a broad range of buffer conditions. The lipids in direct contact with the transmembrane region of a protein are often referred to as the “annular lipid belt” and undergo exchange with the bulk lipids of the membrane. In the absence of any selectivity between lipids and protein, the annular belt will largely reflect the composition and properties of the surrounding membrane. Detailed insights into the nature of non-selective lipid interactions are however hard to find. Bacterial Aquaporin 0 (Aqp0) provides one of the few high-resolution structures that reveals a full annular lipid belt [3,4]. The 2D electron crystallography structure shows that the Aqp0 tetramer is surrounded by lipids that contact the protein both through nonpolar alkyl chains and polar head-groups, adapting to the surface of the protein to form a tight seal around the transmembrane region. As a result, these lipids often occupy similar sites on each protein subunit. MD simulations of proteins in model membranes offer the most detailed insights into the determinants of low-selectivity lipid interactions [5]. Comparisons between the

available structures of Aqp0 and its annular lipid belt with all-atom MD simulations of the protein in a homogenous lipid bilayer show that the lipids adopt essentially the same positions with respect to the protein [6,7]. MD experiments also indicate that the residence times of individual lipids at these annular sites are short due to frequent exchange with the bulk lipids in the membrane. In a recent study, coarse-grained simulations in phosphatidylcholine bilayers were employed to compare the frequencies with which lipids occupy the annular sites in over 2000 membrane proteins (Figure 1) [8]. The approach correctly distinguished the selective lipid binding sites in the inward rectifier K⁺ channel Kir3.2 as well as the general preference of Aqp0 for non-selective contacts, although a few moderately preferred subsites could be observed (Figure 1). These studies demonstrate that MD is capable of assessing the selectivity of lipid contacts for a large number of membrane proteins.

Observing functionally important lipids

When high concentrations of detergents are present, such as for crystallization of detergent-solubilized membrane proteins, nonselective lipid interactions are usually lost because they are outcompeted by the detergents. As a result, the presence of lipids in X-ray crystal structures often indicates selective or particularly strong interactions. Selective lipid binding sites have been described for a variety of proteins [9,10]. For example, the inward rectifying potassium channel Kir2.2 has phosphatidylinositol molecules sandwiched between each monomer in its tetrameric structure and held in place by conserved positive residues [11,12]. Recruitment of the preferred lipid phosphatidylinositol 4,5-bisphosphate induces well-defined local rearrangements, suggesting a lipid-selective mechanism [11,12]. The COPI

machinery protein p24 recognizes a single sphingolipid species using a conserved binding site, which has been proposed to induce oligomerization and regulate vesicle transport in the ER [13]. Similarly the β 2-adrenergic receptor (β 2R) was shown to require cholesterol at the dimer interface to preserve its native structure [14,15]. Interestingly, a recent survey of over 100 high resolution structures of membrane proteins with bound lipids did not arrive at any common molecular determinants for lipid binding sites, precluding the reliable prediction of selective lipid interactions in all but a few instances [16,17]. Therefore, insights into the connection between structural and functional implications of lipid binding are for the most part limited to protein-specific, highly selective interactions that can be maintained even under delipidating conditions.

There is however accumulating evidence from biophysical and biochemical studies that low affinity protein-lipid interactions may also represent selective and biologically important interactions. A prominent example is the mechanosensitive channel of large conductance (MscL). By introducing tryptophan residues at different sites in the transmembrane region and measuring the fluorescence quenching conferred by different lipid environments, lipid binding sites with intermediate specificity in the grooves between the protomers were identified. Anionic lipids preferentially interacted with a nearby cluster of positively charged residues, but neutral zwitterionic lipids could also occupy those sites when anionic lipids were absent [18]. In the crystal structure of the functionally but not structurally related *E. coli* mechanosensitive channel of small conductance (MscS), lipid alkyl chains occupy the grooves between protomers, with similar effects on the fluorescence quenching of MscS tryptophan mutants. MD simulations suggest a selective recruitment of

phospholipids from the membrane to these sites to support the closed state of the channel [19].

A fluorescence reporter based approach recently revealed that in addition to requiring cholesterol for dimerization, β 2R also binds anionic lipids to modulate its activity [20]. Here, the presence of lipids with negatively charged head-groups modulates ligand affinity and promotes receptor activation via an allosteric mechanism, although the location of the interaction site is unknown. Activation of β 2R was also found to occur independently of lipid binding, albeit at a slower rate, suggesting that the observed lipid preferences serve to fine-tune its activity.

Another way to probe the role of annular lipids is co-purification of membrane protein complexes together with their surrounding lipid environment using styrene-maleic acid copolymers [21]. Here, styrene-maleic acid lipid particles (SMALPs) remove the membrane protein from the native membrane, and MS-based lipidomics can be used for the “a priori” identification and quantification of lipids that are co-extracted with a specific membrane protein. Analysis of the SMALPs containing the bacterial Sec translocase complex shows that it preferentially associates with negatively charged lipids, which in turn have a positive effect on functionality [22]. These studies highlight the possibility that more transient, annular lipid interactions can bear functional and structural relevance.

Mass spectrometry captures the entire selectivity range of lipid interactions

Despite an increasing appreciation for the functional importance for transient and weak lipid interactions, their structural implications remain challenging to observe. Here, MS can provide important insights, as it allows us to trap and study isolated protein-lipid complexes. “Soft” ionization techniques such as electrospray ionization

help preserve the structures and interactions of protein complexes as they are transferred from solution to the gas phase, *i.e.* the vacuum region inside the mass spectrometer. Once the solvent is removed, these complexes can be subjected to intact mass measurements or collisional dissociation to release their individual components. Maintaining a protective detergent or lipid environment around membrane proteins during the transition into the gas phase helps to retain non-covalent interactions, mimicking the membrane environment. Dissociation of the weakly bound components through collisions with inert buffer gas releases the protein complex, and interactions with associated lipids can be detected by measuring the mass of the intact complex. Carefully adjusting the strategies for the introduction and release of protein complexes preserves a wide range of lipid interactions for interrogation in the gas-phase by MS.

From an MS perspective detergents become more difficult to dissociate from the protein complex the more they resemble biological lipids [23]. Thus, by selecting detergents that do not resemble lipids and interact only weakly with the membrane protein, it is possible to optimize the gas-phase collisional dissociation to remove detergent micelles while retaining residual lipids that have remained attached to the detergent-solubilized protein during purification. These lipids are therefore likely candidates for structurally or functionally significant interactions [9].

The high complexity of biological membranes however makes it challenging to identify bound lipids in intact protein complexes. Many naturally occurring lipids have similar masses, and are therefore progressively difficult to resolve with the increasing size of protein complexes. This problem can be overcome to some extent by recent developments in high-resolution mass spectrometry, such as the Orbitrap technology. Widely used in proteomics studies to separate individual components in

complex peptide mixtures recent modifications have made these instruments amenable to the study of large protein assemblies [24], and most recently intact membrane protein complexes (Figure 2) [25]. With the increased resolving power, it is possible to determine the exact masses of multiple lipid adducts co-purified with the sugar exporter semiSWEET for example, allowing the direct identification of the bound cardiolipins with a preference for longer chain lengths than the average found in the *E. coli* membrane. This finding provides direct evidence for selective recruitment of lipid species to the local membrane environment by a membrane protein.

MS does not only help to identify lipid interactions, but can also reveal their functional and structural impact (Figure 3). The ABC transporter TmrAB is a striking example of a complex that retains a large number of lipids following purification. MS analysis was only possible following prolonged delipidation with strong detergents [26]. Interestingly, in the cryo-EM structure a large mass of unassigned density was observed surrounding the transmembrane segment [27]. Analysis from both lipidomics and native MS allowed assignment of this density to negatively charged phospholipids and Lipid A derivatives bound to high-affinity sites in the annular region and the substrate binding pocket. ATPase activity and lipid depletion could be observed simultaneously in MS experiments, suggesting that TmrAB can act as an ATP-dependent lipid flippase [26].

The addition of exogenous lipids for MS analysis of membrane proteins can be used to investigate interactions with a defined set of lipids, as well as to reconstitute any transient interactions that are typically lost by delipidation during purification. By trapping such transient interactions inside the mass spectrometer, it is possible to

distinguish lipid-bound and lipid-free protein populations. Collision-induced unfolding and ion mobility MS of different sub-populations can then be applied to probe how different lipids affect protein structure and stability. Using this approach, it was recently shown that phosphatidyl glycerol (PG) and cardiolipin stabilize the ammonia channel AmtB [28]. The structure of AmtB crystallized in the presence of PG mixture revealed five lipid molecules bound to the AmtB trimer via H-bonds with the ionic head-group, providing a rationale for the stabilization of AmtB against gas-phase unfolding by selectively bound lipids.

The protein-lipid interactions that persist in the gas phase are not limited to instances with selective binding. Strong evidence for the ability of MS to probe even completely non-discriminating interactions comes from the use of nanodiscs as vehicles for transferring membrane proteins from a lipid bilayer into the gas phase. This can be achieved by inserting membrane proteins into lipid nanodiscs, which are nanoscale bilayers held together by an apolipoprotein belt. Nanodiscs self-assemble when detergent is removed from the reconstitution mixture of scaffold protein, membrane protein, and lipid [29]. The composition of the lipid bilayer is defined by the lipids added to the reconstitution mixture. Membrane proteins residing in a native-like bilayer can be released by high energy collisional activation [30]. Moreover, peripheral membrane proteins retain endogenous lipids after release from nanodiscs [31]. Low-energy dissociation of nanodiscs was investigated recently using the high-resolution Orbitrap mass spectrometer. With the help of Bayesian deconvolution software to resolve the arising spectral complexity [32], it was found that the distributions of the protein-lipid complexes released from nanodiscs are significantly affected by the activation energy [33]. At low collision energy, membrane proteins

are released with a large number of bound lipids, which correspond to the average number of lipids in the protein's annular shell. At increasing collision energies, the annular lipids largely dissociate, leaving only the lipids that are bound to the protein via polar head group contacts. AqpZ, a homologue of Aqp0, was found to retain a lipid annulus of approximately 40-60 lipids, which is in good agreement with the number of annular lipids identified by 2D electron crystallography [3,6,7]. In the case of AmtB, the protein retains a set of around nine lipids after stripping the annular belt, which agrees with MD simulations for the number of lipids interacting via ionic contacts. These studies demonstrate that the full range of protein-lipid interactions can be maintained in the gas phase, allowing us to distinguish co-purified endogenous lipids, preferentially bound lipids, and weakly interacting annular lipids (Figure 3).

A sliding scale of protein-lipid interaction specificity

All membrane proteins are exposed to an array of lipids with differing physical properties, and as outlined above, activity is in some cases dependent on the availability of specific lipids. While highly specific lipid requirements can in extreme cases serve as a lateral regulator of protein activity, the vast majority of membrane proteins will be exposed to multiple lipids with different physical properties. Combining evidence from X-ray crystallography, MS, MD and other approaches, we find that most membrane proteins, although not critically dependent on a single lipid species, are likely to exhibit some degree of lipid selectivity along a sliding scale from highly specific to largely non-discriminating. On the far end of the scale, we find proteins such as Kir2.2 and p24 that are crucially dependent on binding individual lipid molecules at well-defined sites. In the case of Kir2.2, the protein requires

specific sites to be occupied by matching lipids to ensure its proper function [11,12], while the p24 protein senses the presence of sphingolipids to direct vesicle transport [13]. In the intermediate region of the scale, we find proteins that display lipid binding preferences but may not be dependent on these interactions for their biological function. An example is TmrAB, which appears to actively select anionic lipids to populate part of its annular lipid binding region using large number of moderately selective sites [26]. The lower end of the specificity scale is populated by proteins that are able to fulfil their biological functions without any preferences regarding membrane composition. Nevertheless, even the proteins without any specific lipid requirements may still contain lipid binding pockets or sites that need to be occupied by lipid molecules.

The membrane proteins highlighted here have been investigated by a variety of biophysical approaches and together show that low-specificity interactions often involve sites with increased affinity for charged lipid head-groups. Placing a positively charged residue in close proximity allows accurate lipid positioning, resulting in longer lipid residence times and the possibility of preferential binding of a subset of lipids at the target site. This enables the protein to tailor its membrane environment according to its structural and functional requirements without being critically dependent on the presence of a specific lipid. It is tempting to speculate that the moderate selectivity of proteins such as MscL, TmrAB and β 2R towards negatively charge lipids can be attributed to the use of head-group charges to direct and maintain lipid binding in functionally important positions. More generally we propose that the sliding selectivity scale of lipid binding described here will act to fine-tune biological activity, and in turn membrane proteins will shape their surrounding lipid environment to suit their function.

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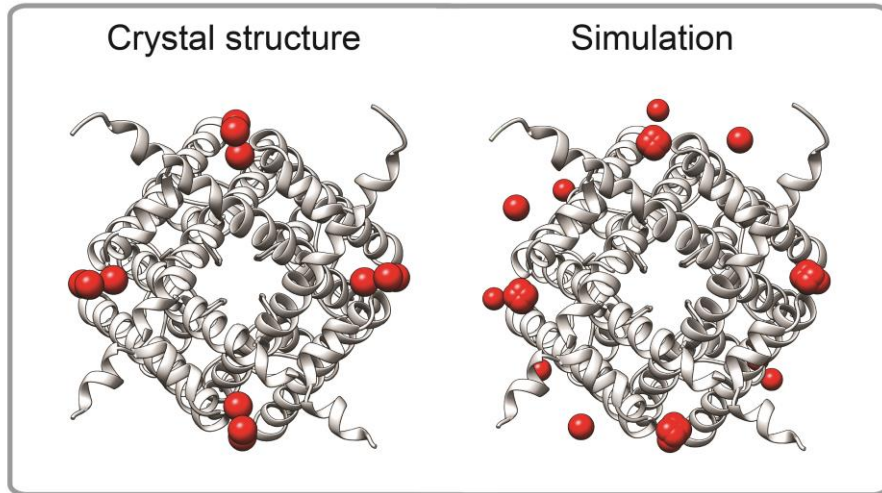
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Figures

Kir3.2



Aqp0

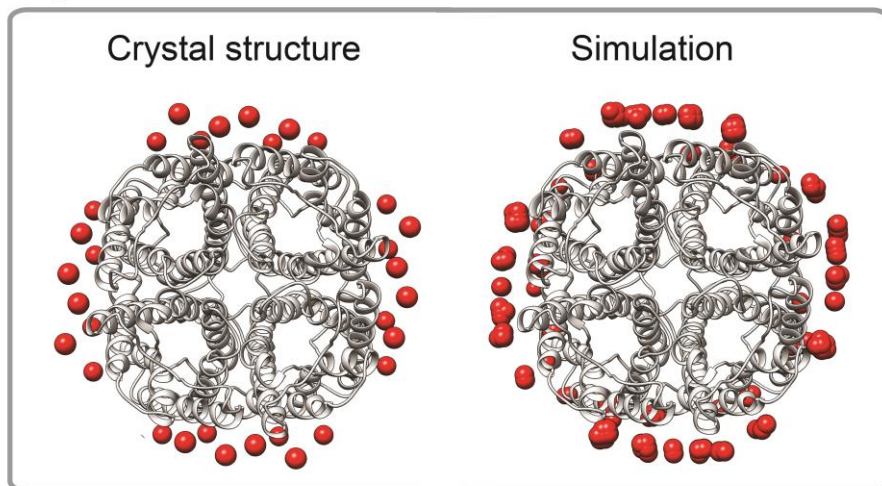


Figure 1. Lipid fluctuations in MD simulations correspond well with lipid locations in crystal structures. Top: The structure of the transmembrane segment of the K⁺ channel Kir3.2 viewed from the cytoplasmic side shows four selectively bound lipids around the central pore, which were found to be occupied with high frequency in coarse-grained MD simulations. The intracellular domains are omitted for clarity. Aqp0, on the other hand, shows an even lipid distribution around the entire transmembrane segment when viewed from the cytoplasmic side, with only a few sites exhibiting slightly higher occupation frequencies. Figure adapted from [8].

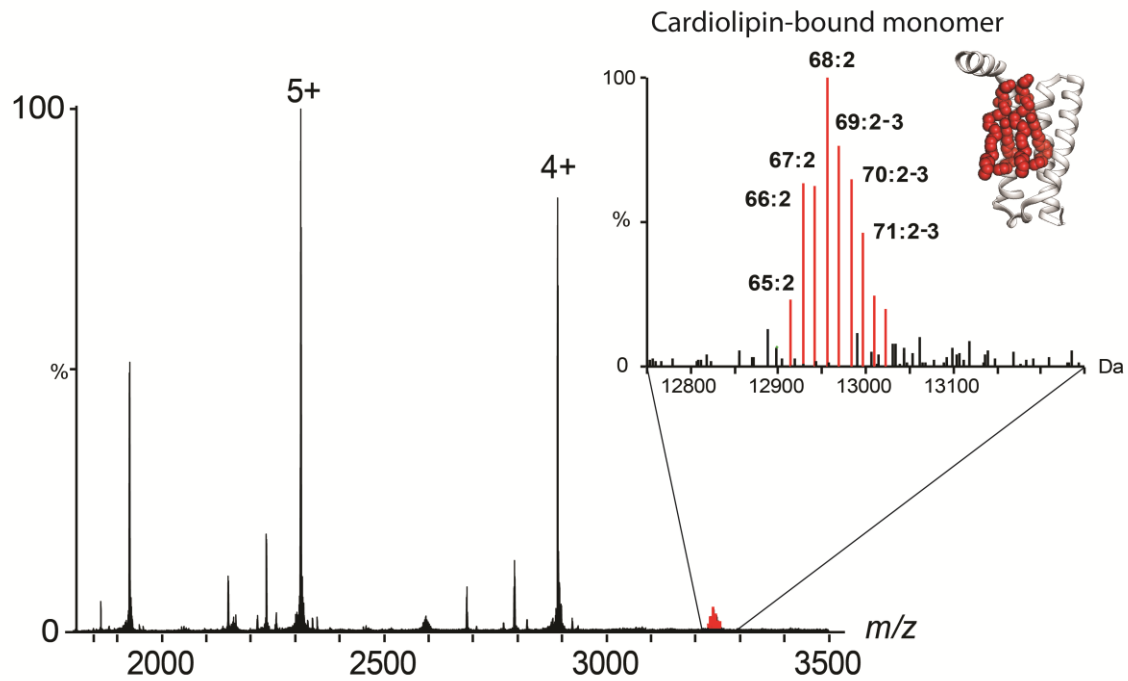


Figure 2. High-resolution Orbitrap MS of membrane proteins. A mass spectrum of the sugar exporter semiSWEET shows a cardiophilin-bound monomer population. The spectrum was recorded using nano-electrospray ionization following release from DDM micelles. The modified Orbitrap mass spectrometer allows the identification of individual acyl chain configurations (insert, numbers indicate the total number of carbons in the lipid acyl chains), revealing a preference for lipids with longer chain lengths. Figure adapted from [25].

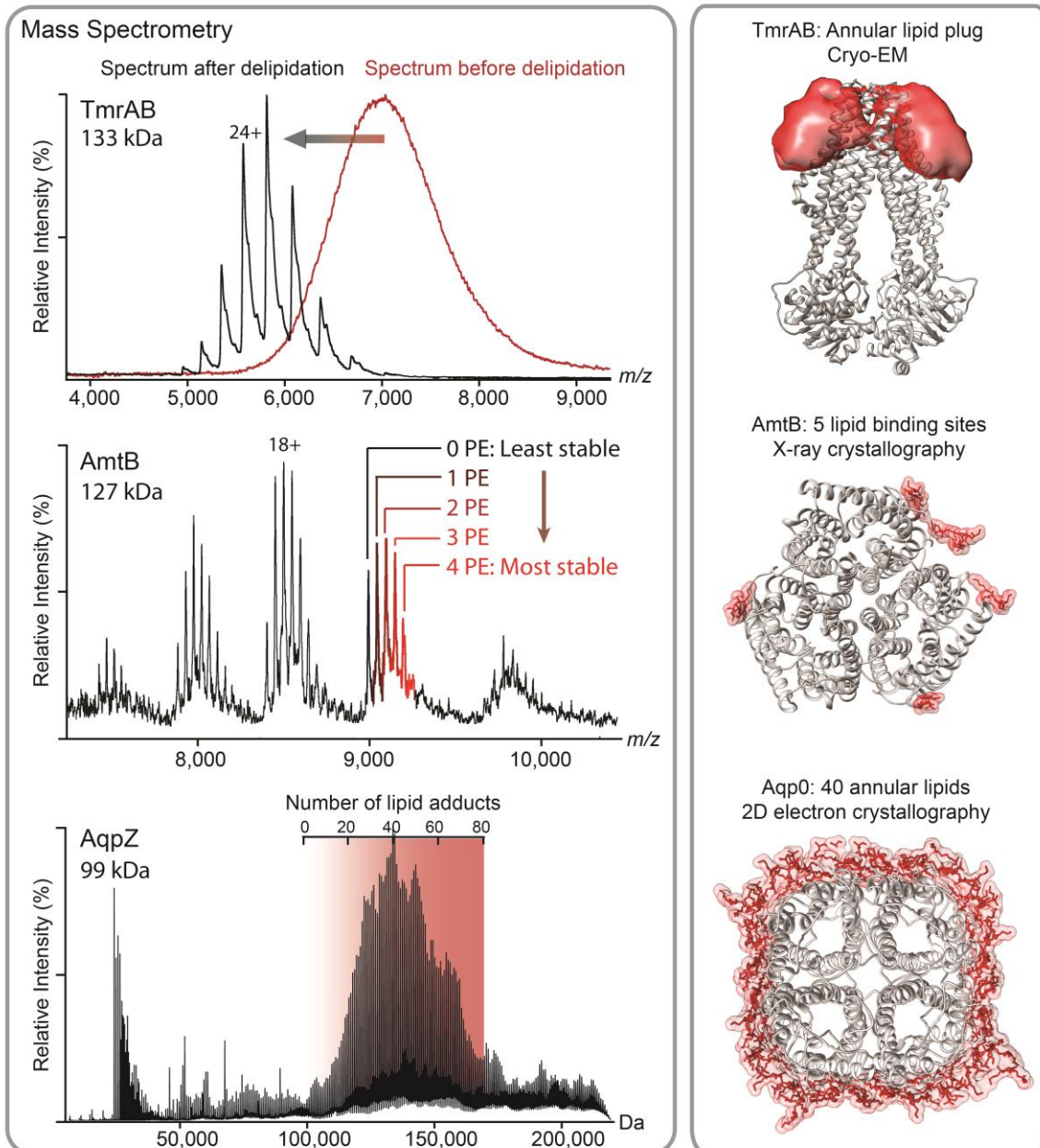


Figure 3. MS captures a range of protein lipid interaction selectivity. Top: nESI-MS spectra of TmrAB show the presence of a large number of lipid adducts which can be removed in solution using strong detergents. The large electron density observed around the transmembrane segment with cryo-EM likely represents this tightly bound lipid subset. Middle: Addition of negatively charged lipids to AmtB results in the formation of protein-lipid complexes. As the number of bound lipids increases, the complex becomes increasingly stabilized against collisional unfolding. The crystal

structure obtained in the presence of PG shows five binding sites with high affinity for negatively charged lipids. Bottom: Following release from lipid nanodiscs, AqpZ retains up to 80 weakly bound lipids, in good agreement with the annular belt of 40-81 lipids observed by 2D electron crystallography.