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Perspective

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Different Modes of Lipid Binding to Membrane Proteins Probed by Mass Spectrometry

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ABSTRACT: The realization that the lipid environment is crucial for maintaining the structure and function of membrane proteins prompts new methods to understand lipid interactions. One such method, mass spectrometry, is emerging with the potential to monitor different modes of lipid binding to membrane protein complexes. Initial studies monitored the addition of lipids and deduced the kinetic and thermodynamic effects of lipid binding to proteins. Recent efforts however have focused on identifying lipids already present, explicitly in plugs, annular rings, or cavities. Lipids that bind within these orifices to membrane proteins will have higher residence times than those in the bulk lipid bilayer and consequently can be quantified and characterized by mass spectrometry. In special cases, lipids identified within cavities have been proposed as substrates following activity assays. Alternatively, a gas-phase unfolding protocol can be used to distinguish lipids that are important for stability. These lipids can subsequently be added during crystallization for the characterization of lipid-bound protein complexes. Overall therefore this Perspective provides an overview of recent advances in mass spectrometry, with a particular focus on the distinction of the various modes of lipid binding, and their implications for structure and function as well as new directions that lie ahead.

Membrane proteins are the gatekeepers of the cell, mediating the traffic of solutes in and out of the cell and translating the action of extracellular stimuli into function. Given these key roles, membrane proteins represent a prime target for drug discovery. Being present in hydrophobic and highly dynamic complex bilayers, however, renders membrane proteins difficult to study *in vitro*. While spectacular advances have been made using X-ray crystallography,[1] the conformational dynamics within membrane proteins are often difficult to assess. Considerable success has been achieved with NMR spectroscopy for individual membrane proteins and small complexes[2,3] and in the application of FRET measurements with suitably labeled proteins.[4,5] Recent developments in cryo-EM are also yielding unprecedented resolution for membrane proteins with near atomic resolution of complexes prepared in detergent micelles.[6] Defining small molecules within atomic structures however remains problematic due to the difficulties in distinguishing detergent molecules from lipids and in defining dynamic hydrocarbon side-chains and buried head groups.[7]

Mass spectrometry (MS) is beginning to contribute to the structural characterization of membrane proteins, primarily by exploiting gaps left by other structural biology approaches. MS has already been applied to the identification of post-translational modifications (PTMs) and is uncovering the sequences of the membrane proteome.[8] Secondary and tertiary structure determinations of membrane proteins have also been achieved by coupling chemical modification and labeling of complexes, followed by proteolysis and MS recorded under denaturing conditions. Specifically, hydrogen−deuterium exchange[9,10] and hydroxyl radical footprinting[11] as well as chemical cross-linking[12,13] have been employed to uncover conformational changes induced by protein−ligand binding, protein−protein interactions, and PTMs.

A relatively new approach that enables a direct "view" of dynamic membrane assemblies involves recording mass spectra of intact complexes released in the gas phase from detergent micelles formed in solution. This approach can allow label-free real-time snapshots of noncovalent interactions. When coupled with ion mobility (IM), a technique that provides a rotationally averaged collision cross-section (CCS) of proteins and their complex, a further dimension becomes possible.[14−16] Following addition of ligands or substrates, IM-MS enables a direct assessment of the effects of small molecules on membrane protein conformations in the gas phase.[17,18] In this perspective, we will focus on recent applications of MS to intact membrane assemblies, beginning with a historical overview of the developments, followed by a classification of protein−lipid interactions and ending with a discussion on future directions.

DETECTING INTACT MEMBRANE ASSEMBLIES

Early attempts to analyze intact membrane proteins in the gas phase were motivated by the prediction that the vacuum offers a suitable hydrophobic environment for such biomolecules.[19] The challenge at that time, however, was to find a method to transfer membrane protein complexes intact from solution into the gas phase. The first vehicles investigated for this purpose were large unilamellar vesicles into which small hydrophobic peptides were reconstituted.[20,21] MS analysis of TFE-induced vesicle dispersions revealed the selectivity of lipid binding to the bacterial K(+) channel KcsA reconstituted into vesicles of variable lipid composition. The resulting mass spectra showed noncovalent complexes of KcsA and phospholipids with preferential binding to the anionic phosphatidylglycerol (PG) and, to a lesser extent, the zwitterionic phosphatidylethanolamine (PE), both of which are abundant bacterial lipids. These preferred interactions may reflect the differences in affinity of these phospholipids for KcsA in the membrane. Extending this protocol to study the intact membrane protein complex however did not preserve subunit interactions within the

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tetrameric K^+ channel KcsA, presumably due to the very harsh MS conditions required to disrupt proteoliposomes while maintaining the intact complex.[22]

As a result, focus turned from artificial bilayers toward the use of detergents to introduce membrane protein complexes. High concentrations of detergents however can have a deleterious effect on the quality of mass spectra recorded due to the large background of detergent ions generated upon ESI, possible signal suppression, and adduct formation.[23] To circumvent this problem, nonionic detergents were chosen since they can be tolerated more readily in electrospray than ionic detergents.[24] First studies, in which the detergent concentration was reduced below the critical micelle concentration (CMC), were reported for the dimeric multidrug transporter EmrE and enabled the detection of drug molecules and individual protein subunits.[25] However, maintaining interactions in the gas phase between transmembrane subunits and small molecules, as well as soluble and transmembrane subunits, remained elusive.[26,27]

While it was intuitive to remove detergent prior to MS, to prevent signal suppression, it was the realization that the detergent micelles themselves both stabilize and protect membrane assemblies that transformed the field.[28] Rather than depleting the detergent, as had been attempted previously, the detergent concentration was maintained above the CMC during electrospray introduction of the protein complex. This is in line with established structural biology approaches, commonly used to characterize membrane proteins, which rely on the use of detergent micelles above the CMC to solubilize and maintain stability outside of the bilayer. For MS experiments the protein complex is released from the detergent micelle only once in the gas phase. In accord with this protective, shielding role it was shown previously that aspects of micellar structure are maintained in the gas phase.[29−31] Moreover, amino acids and soluble proteins can remain encapsulated in reverse micelles upon transition from solution to the vacuum.[30,32,33]

Maintaining the detergent concentration above the CMC in solution and subsequently removing bound detergent molecules by collision with inert gas molecules in the mass spectrometer was the key step in preserving the interactions between the soluble and transmembrane subunits of the ABC transporter $BtuC_2D_2$.[28] The discovery of these conditions enabled demonstration of the cooperativity of nucleotide binding to $BtuC_2D_2$ and paved the way for a whole new research field. As a result of this ability to use MS to determine exact stoichiometries, it became possible to observe peptide and drug binding to multicomponent complexes, containing both membrane and soluble proteins.[13,34−37]

DETERGENT MICELLES PROTECT MEMBRANE PROTEINS

Given that the transformative step was to use micelles to protect complexes, the question arises as to how this affects the ionization process and folded structure of membrane proteins. A key observation was that the membrane protein complexes analyzed to date typically have lower charge states than soluble proteins of comparable surface area. However, if the surface area attributed to transmembrane regions is subtracted from the total surface area, membrane proteins and soluble proteins have similar average charge states.[38] Hence, the presence of the micelle surrounding the hydrophobic parts of a membrane protein appears to protect the latter during the ionization process. Moreover the formation of low charge states could also protect membrane protein complexes from charge-driven unfolding, at least while in the micelle.

Of interest are molecular dynamics (MD) simulations that probe the effect of water evaporation on micelle-encapsulated membrane proteins in vacuum.[39,40] Upon evaporation of water during the phase transition, hydrophobic interactions at the micellar surface are increased due to the enhancement of electrostatic interactions between detergent head groups and a reduction in hydrogen bonding between the detergent and the solvent.[39] As a result the number of hydrogen bonds in the encapsulated transmembrane protein are not altered drastically (Figure 1). This is in contrast to the situation for dehydration

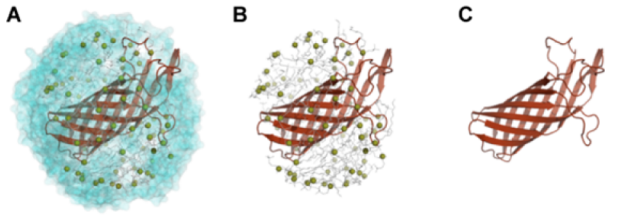

Figure 1. MD simulation of the transition of a proteomicelle from solution to vacuum. Rendition of the N-terminal domain of *E. coli* outer membrane protein A (OmpA171) in a hydrated DPC micelle (A), upon transition of the proteomicelle to vacuum (B), and the detergent-free protein after collisional activation in the gas phase (C). Atomic coordinates were taken from the end of a 150 ns MD simulation in the gas phase.[39]

of soluble proteins, wherein an increase in intramolecular H-bonds occurs which in turn may lead to structural rearrangements.[41] These MD simulations therefore suggest that during transfer from water to vacuum membrane proteins are protected, to a greater extent than soluble proteins, from major structural changes by virtue of the surrounding micelle.

Once proteomicelles have been ionized and transferred into vacuum, detergent molecules surrounding the proteins are removed by thermal agitation, via collisions with inert gas molecules (Figure 2). As the micelle is removed and the transmembrane regions of the complex become exposed, maintaining folded structure becomes increasingly difficult. Recent studies, combining IM and MS, revealed that the release of detergent molecules during collision-induced dissociation (CID) can help to protect their conformation.[42,43] This is likely akin to the way in which salt additives stabilize soluble protein complexes.[44,45] The increased internal ion energy gained upon collisional activation of micelle-encapsulated membrane proteins is dissipated via disruption of detergent aggregates and detergent-protein assemblies (Figure 2). Dissociation of detergent molecules is driven primarily by evaporation, leading to neutral losses, and to a lesser extent by fission events, which effectively strip charges from the protein. Neutral losses of the detergent will reduce the internal energy of the remaining assembly, maintaining it below the energy required for conformational changes. Once the detergent is removed, additional collisional heating will lead to structural changes in the membrane protein complex. Hence, a balance needs to be found between "cleaning-up" the membrane protein complex while avoiding its overactivation. The energy required to attain this balance will depend primarily on both the nature of the detergent and the structure of the proteomicelle.

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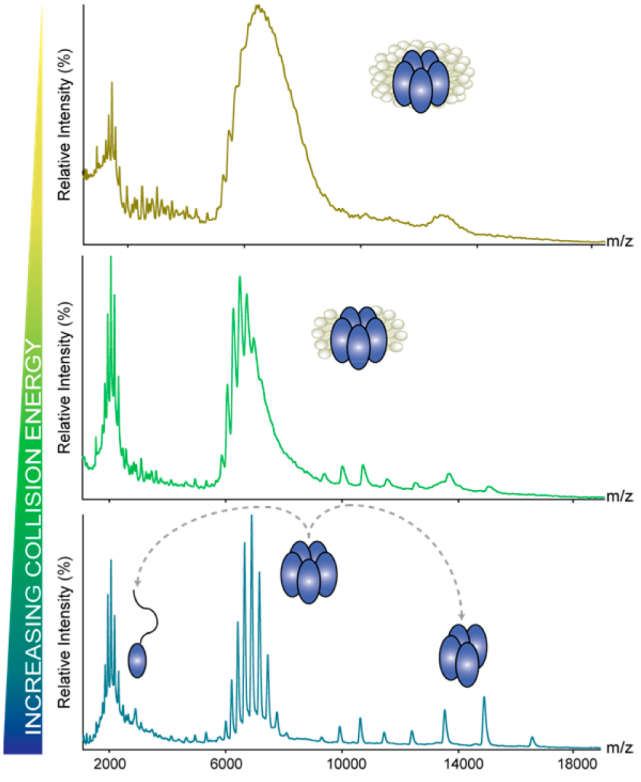

Figure 2. Membrane protein ejected from a detergent micelle. nanoESI mass spectra, recorded under increasing collision energy, of the pentameric ligand-gated ion channel ELIC in DDM detergent micelles.[46] At lower energies (120 V, top), the protein is still trapped in the detergent micelle, and as the activation energy is increased (140 V, middle), gradual loss of detergent molecules is reflected by an increase in the resolution of the mass spectrum of the stripped complex. At high energies (180 V, bottom), the complex is disrupted via CID to give highly charged monomers and "stripped" tetramers at higher m/z than the pentamer. Spectra were acquired on a modified Q-TOF2,[47] instrument conditions were as follows: capillary voltage 1.8 kV, cone voltage 50 V, extraction voltage 10 V, source backing pressure 7 μbar, and a collision cell pressure of 3 MPa.

Similar to the workflow used in X-ray crystallography,[48] a systematic detergent screen is necessary to achieve optimal MS conditions,[46] the selection of the appropriate detergent being a protein specific phenomenon. Short chain polyoxyethylene glycol detergents, as well as dimethylpolydecylamine-*N*-oxide, show a general tendency to require lower activation energy in order to obtain resolved mass spectra of membrane proteins.[49] However, shorter chain detergents often destabilize membrane proteins in solution compared to longer chain ones.[50] Saccharide detergents commonly used in structural biology, such as *n*-dodecyl-β-D-maltoside (DDM) or *n*-decyl-β-D-maltoside (DM), can perturb the folded states of low molecular weight membrane proteins, but may be used in conjunction with charge reducing agents to yield folded membrane protein complexes.[51] Alternatively, the use of amphipathic surfactants instead of classical detergents in some cases was shown to maintain folded structures of monomeric proteins in the gas phase.[52,53]

Interestingly, irrespective of the detergent micelle, one of our earliest realizations was that the masses of our complexes invariably corresponded to lipids bound to the membrane proteins. This raises important questions: How would we characterize the lipids that bind to these complexes? Are they simply a product of the isolation procedure, transiently associated with a membrane protein, or are they an integral part of the structure?

CLASSIFYING PROTEIN–LIPID BINDING INTERACTIONS

The extent and proximity of lipid interactions with membrane proteins has been divided into three main classes.[54] Those that constitute the first motionally restricted shell directly in contact with the transmembrane regions are referred to as annular lipids (Figure 3). Despite the fact that these annular lipids are

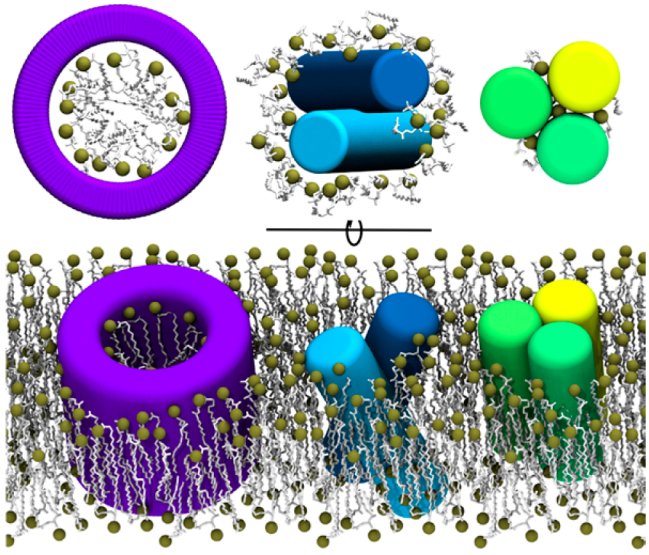

Figure 3. Distinctive modes of lipid binding to membrane proteins. Lipids can serve as plugs in channels and rotary pumps (left), as annular shells surrounding membrane proteins (center), or as integral components in structures present in specific clefts or binding sites in the trans-membrane regions and/or between membrane protein subunits (right).

in constant exchange between the surface of the membrane protein and the bulk of the plasma membrane, a given protein complex is constantly shielded by a layer of annular lipids. Their fast dynamic exchange with the environment however makes these lipids challenging to capture with classical structural biology approaches.

The second class of lipid interactions consists of those that bind more tightly to certain clefts and grooves within a membrane protein or its complex. These lipids are not subject to the dynamic exchange experienced by annular lipids and are often termed "structural lipids". Being less easily displaced by detergent micelles than the bilayer bulk lipids or the annular lipids upon extraction of proteins from the membrane, structural lipids are more likely to be detected in crystal structures provided sufficient resolution is attained.[55]

Distinct from the annular and structural lipids is the third class of lipids, which can take the role of specific substrates. An example of this class of lipid is seen with lipid flippases that bind and actively move lipids across the bilayer.[56]

While some overlap of lipids between these three categories might be anticipated, with annular lipids becoming structural lipids or structural lipids acting as substrates, such a classification is useful for selecting the appropriate detergent micelle, bicelle or liposomal environment for detecting their presence. Moreover, understanding the residence times of lipids

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in these different environments will lead to a greater understanding of their functional and structural roles in membrane protein complexes.

SELECTIVITY VERSUS LIPID ABUNDANCE

A key point to note is the experimental observation that the residence times of various lipids within different membrane proteins is not necessarily governed by the lipid abundance within a given membrane. This absence of a direct relationship between selectivity and abundance has been demonstrated in various studies. For example, in the X-ray structure of the membrane rotor of the V-type ATPase from *E. hirae*, 20 PG lipids were modeled into the electron density maps since PG is the most abundant phospholipid in this species.[57] Subsequently it was revealed by MS of the intact complex combined with quantitative lipidomics and proteomics, that precisely 10 cardiolipin (CL) molecules occupy the cavity within each membrane rotor.[35] Further examples of selective lipid binding include activation of the inward rectifier K(+) channel Kir2.2, by phosphatidylinositol-biphosphate (PIP2), and activation of the respiratory complex in bacteria by CL, both of which are low abundance bilayer phospholipids.[58,59]

An important conclusion from these studies is that, wherever possible, structural and biochemical studies should attempt to preserve the natural lipid environment in which the membrane proteins reside. Moreover such studies should also consider not only the role of structural lipids but also the presence of annular lipids and the potential for lipids to act as substrates. These three different lipid-binding scenarios can be distinguished by MS as we illustrate below.

USING MS TO DISTINGUISH PROTEIN–LIPID BINDING MODES

After the revolutionary finding that micelles protect complex membrane assemblies in the gas phase, determining subunit stoichiometry of membrane protein complexes became a relatively "straightforward" task. Classical biophysical solution techniques are hindered by the presence of detergent micelles, whereas MS permits the exact mass of the complex to be determined, devoid of the detergent micelle. Moreover, it is possible to detect binding of small molecules and drugs to membrane proteins by changes in mass and in some cases to determine the kinetics and thermodynamics of binding, using either real time experiments or time-resolved mass spectra.[60] These attributes have major implications for drug discovery but are not discussed further here. As the main focus of this Perspective, we describe how MS of intact complexes has enabled the distinction and classification of different types of membrane protein–lipid interactions (Figure 3).

The first type of lipid binding involves the identification of endogenous lipids purified with membrane protein complexes. The main obstacle to the identification of endogenous lipids lies in the fact that membrane proteins are extracted from natural bilayers using detergents. These detergents act by competing with lipid–lipid and lipid–protein interactions to replace lipids and solubilize membrane proteins while preserving subunit interactions. Consequently, displacement of selectively bound native lipids could occur.[61] Careful choice of detergent and extraction protocols is therefore crucial in maintaining binding of endogenous lipids to proteins. Preserving these lipids however can result in considerable heterogeneity leading to mass spectra that are impossible to assign (Figure 4A). Further removal of native lipids to obtain a homogeneous protein may perturb folding and impair activity.

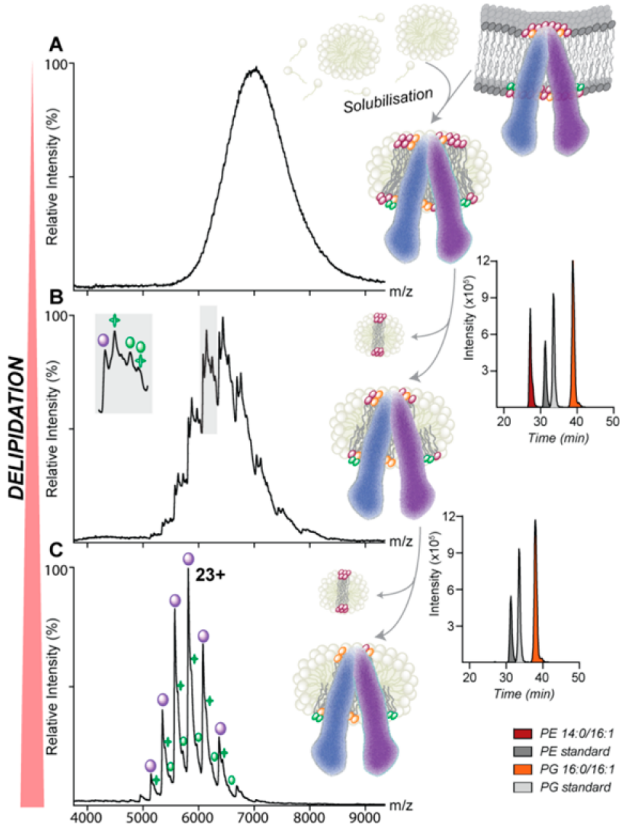

Figure 4. Monitoring lipids associated with an ABC transporter. Mass spectra of purified TmrAB before (A) and after progressive delipidation (B and C). Increased charge-state resolution is visible with extended delipidation. Insets show the extracted ion chromatograms of representative PE and PG species quantified before (upper) and after (lower) delipidation, revealing the depletion of PE lipids whereas the amount of bound PG remains constant. (C) Charge states of the *apo* complex and the binding of the 1.4 and 2.1 kDa species are indicated (purple circles, green cross and circle respectively). Figure adapted from ref 64.

If it is possible to remove the vast majority of endogenous lipids, while retaining solubility and activity in a detergent micelle, it becomes feasible to monitor binding characteristics of selected lipids as shown in the next section.

BINDING TO EXOGENOUSLY ADDED LIPIDS

The multidrug ABC transporter P-glycoprotein (P-gp) retains activity in DDM detergent micelles, and despite the background of detergent molecules that continuously efflux through the pump, individual lipid binding could be monitored by MS following addition of exogenous lipids.[36] By monitoring the formation of lipid-bound states over a period of time, it was possible to determine an apparent affinity, which led to the deduction of kinetic and thermodynamic parameters. Seven different phospholipid classes were investigated; results showed a higher binding affinity to negatively charged lipids than to zwitterionic lipids. Interestingly, affinity was affected much less by changes in acyl chain unsaturation than polarity. This is consistent with other membrane protein systems in which preferential binding to negatively charged lipids has been reported, driven by electrostatic interactions between the lipid

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headgroups and side chains of arginines and lysines.[55,62] For lipids with the same net charge, binding of P-gp to smaller lipid molecules was more favorable than for bulkier ones, likely consistent with changes in diffusion.

From a practical viewpoint, care should be taken when titrating with exogenous lipids, since the solution conditions might distort lipid-binding preferences. For instance, higher detergent concentrations will lower the lipid binding capacity of the membrane protein, analogous to the situation in solutions of high ionic strength. Therefore, the thermodynamic values attained reflect apparent affinities that can be used for direct comparison of the affinity of different lipid species to a given membrane protein system. Based on this premise, it was possible however to conclude that differential binding of various lipids to P-gp was driven primarily by the net charge of the headgroup with a secondary steric effect relating to the bulkiness of the side chains.

■ REVEALING ENDOGENOUS STRUCTURAL LIPIDS

In some cases, endogenous lipids remain tightly bound to membrane proteins and are not readily displaced by mild detergents. They therefore coexist in proteomicelles and are observed in mass spectra. Under appropriate conditions, it is possible to determine the exact mass of these lipids as well as their binding stoichiometry. Mass spectra of two different homodimeric ABC transporters, MacB[34] and LmrA,[63] showed the binding of two PE and one CL, respectively. Similarly, two CL molecules were found to bind the heterodimeric ABC transporter LmrCD.[34] In all three cases, lipids were not observed binding to the monomeric subunits, formed via collisional activation, which may imply that the lipids are present at subunit interfaces.

A structural role for lipid plugs in V- and F-type rotary ATPases was proposed following their observation in the MS of the intact membrane rotors.[13,35] The membrane ring movement of rotary ATPases is propagated to the soluble head via the central stalk, which is highly conserved among species. However, the subunit number and architecture of the membrane rotors are diverse, making the diameter of the orifice different between species. To demonstrate the presence of different stoichiometries of bound lipids in the orifice of the ring, quantitative proteomics was performed in conjunction with quantitative lipidomics. While the V-type ATPase from *E. hirae* accommodated 10 CL molecules in its decameric membrane ring, the analogous complex from *T. thermophilus* had only 6 PE molecules within its dodecameric ring. The substoichiometric lipid binding in the second case was proposed to regulate the dual function of the *T. thermophilus* enzyme as an ATP synthase and a cation pump. By contrast, stoichiometric binding in the membrane rotor of the F-type ATPase from *S. oleracea* revealed a diverse population of phospho-, sulpho-, and glyco-lipids.[13] Interestingly, in all cases, the differential mode of lipid binding likely compensates the variation in size of the membrane rings by tightening the membrane orifice to fit the central stalk, with these lipid plugs forming a dynamic seal to aid rotation and maintain the cation gradient.

■ UNCOVERING ANNULAR LIPIDS

It is widely assumed that annular lipids are lost upon extraction of membrane proteins from their native bilayer, most probably because of the harshness of the isolation and purification techniques and the quest for homogeneous protein preparations for structural characterization. The pursuit of homogeneous protein also underlies many protocols adapted for MS. In a recent MS study of TmrAB,[64] a heterodimeric ABC transporter in *T. thermophilus* implicated in multidrug resistance,[65] recombinant TmrAB was purified from *E. coli* using DDM detergent throughout extraction with repeated chromatographic steps.[64] Despite this extensive purification, mass spectra of intact TmrAB revealed unresolved charge states (Figure 4A). Attempts to replace DDM with harsher detergents resulted in precipitation of the protein. The unresolved mass spectral peaks were tentatively assigned to the protein complex with lipids that remain associated throughout purification.

In order to characterize the lipids that remain associated, we devised a controlled delipidation protocol in which the detergent was replenished systematically over a 48 h period in the protein-containing solution. During this "lipid dialysis", we monitored the resulting mass spectra and carried out quantitative lipidomics in parallel. The peaks in the mass spectra became progressively narrow, consistent with a reduction in heterogeneity (Figure 4B,C). These results are consistent therefore with the presence of heterogeneous lipids in the initial proteomicelles, being displaced during prolonged exposure to detergent micelles.

Following this lipid dialysis procedure quantitative lipidomics revealed an average of 13 phospholipid molecules in complex with each purified protein dimer. In addition various lipid A species were also identified associated with TmrAB. Interestingly the cohort of endogenous lipids was consistent in biological replicates following this lipid dialysis protocol. Moreover, comparison of our experimental data with MD simulations in lipid bilayers showed that TmrAB can be surrounded by a lipid belt of ∼20 phospholipid molecules, implying that the majority of this belt is retained following isolation. Contrary to expectations, however, we found that progressive delipidation led to the depletion of all zwitterionic PE lipids bound to the protein. By contrast, levels of negatively charged PG lipids remained constant. This implies that the negatively charged phospholipids are more tightly bound to TmrAB than zwitterionic lipids. It further implies that negatively charged lipids might occupy specific binding sites, consistent with previous reports of a general preference to bind anionic lipids with higher affinity at "hot-spots" on proteins.[62]

Consequently, this study highlights the potential to control the extraction of membrane protein from native bilayers and to use controlled delipidation to retain and subsequently characterize annular lipids in proteomicelles.

■ IDENTIFYING LIPIDS AS SUBSTRATES

A specific class of transporters and channels is able to function as lipid flippases and/or floppases, maintaining bilayer lipid asymmetry and carrying out membrane trafficking.[66] Such proteins use specific classes of lipids as substrates. Methods used to assess their substrate specificity, as well as their mechanism of action, generally rely on indirect assessment, using fluorescent or spin-labeled lipid probes. Interestingly the mass spectra of intact TmrAB showed high molecular mass adducts (1.4 and 2.1 kDa, Figure 4C)[64] that could not be attributed to phospholipids or multiples thereof, but rather indicated the presence of lipid A and its derivatives.

Following direct identification of unlabeled lipid A in purified TmrAB we reasoned that this lipid maybe acting as a substrate. To test this hypotheses, we reconstructed the catalytic cycle *in*

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vitro, in the presence of Mg-ATP and at permissive temperature (>68 °C). The affinity between TmrAB and lipid A under these conditions decreased with time leading to the displacement of the bound glycolipid from the proteomicelle. Furthermore, two protein variants, one that is not capable of ATP hydrolysis and the other that is constrained by cysteine cross-linking, revealed that displacement of lipid A is not only dependent on ATP hydrolysis but also on conformational change. Together these results imply that TmrAB acts a glycolipid flippase in a bilayer context, analogous to its close homologue MsbA in *E. coli*. Moreover, since ATP hydrolysis decreases the affinity for substrate, most probably by inducing conformational changes of the ABC transporter, new insights into the mechanism of lipid flippases were revealed.[56]

■ QUANTIFYING THE STABILITY OF GAS-PHASE STRUCTURES FOLLOWING LIPID BINDING

Having established that it is possible to identify lipids that form an annular ring or plug and to identify the characteristics of lipids that bind proteins with higher affinity, is it feasible to select for lipids that have a structural effect? To do this, a means of investigating the stability induced by lipid binding is required. This was demonstrated recently in a study of three different membrane proteins with varied topologies, oligomeric states, and propensities for lipid binding.[67] Collision-induced unfolding was used to induce unfolding trajectories from which parameters could be extracted to rank the effect of lipid binding on the conformational stability of these proteins (Figure 5). For this purpose, careful choice of detergent was a key step in order to maintain the native structure of the complexes devoid of detergent molecules *in vacuo*. A detergent screen revealed that C_8E_4 gave experimental CCSs in agreement with theoretical ones based on the X-ray structures.

Addition of lipids maintained the folded structures of different lipid-bound states and enabled probable binding sites to be proposed by combining CCS measurements with MD simulations. Moreover, subjecting these complexes to increasing collisional activation induces partial unfolding but does not displace bound lipids. A quantitative comparison of the resistance to unfolding enabled calculation of the extent of stabilization generated by various lipids and was used to rank their effects (Figure 5B). This approach is analogous to other biophysical approaches applied in solution such as thermal denaturation, which reports the average effect for all species present without distinguishing individual binding events. An important point to bear in mind here is that conformational stability, conferred by individual binding events, does not necessarily correlate with binding affinity. The fact that it is possible to observe directly the effects of binding individual lipids however enabled the discovery of lipids important for protein function and structure. Addtion of these lipids led to the first X-ray structure of the ammonium channel AmtB with PG lipids in specific binding sites (Figure 5C).

■ FUTURE DIRECTIONS

Novel MS approaches designed to interrogate the effects of small molecule binding on the structure and stability of membrane proteins are proving critical for uncovering lipids important for stability. A further significant development is the delipidation protocol described here, with its ability to characterize essential lipids post-extraction of protein complexes from biological membranes. Together, these method-

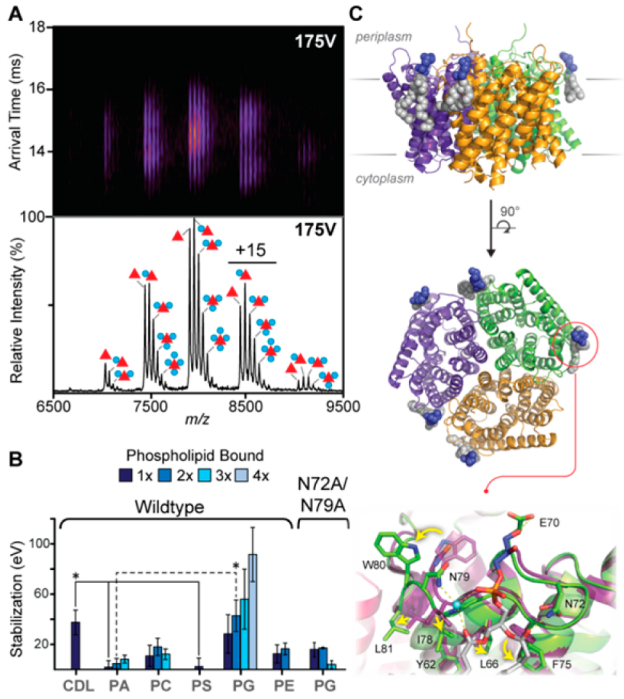

Figure 5. (A) Lipid binding to the trimeric ammonia channel AmtB. IM-MS spectra of AmtB titrated with exogenous PG lipids showing the *apo* protein and the protein bound to up to four PG molecules at 175 V as well as the unfolding data of the different lipid-bound states. (B) Stabilizing effects of various lipid head groups were revealed by monitoring the gas-phase unfolding of AmtB (+15 charge state). Data show that negatively charged CL and PG stabilize the channel significantly against unfolding. A mutant $AmtB^{N72A/N79A}$, engineered to disrupt specific PG binding sites, showed a reduction in the stabilization by bound PG. (C) Crystal structure of AmtB bound to PG molecules (spheres) oriented with the head groups to the periplasmic side. Conformational changes of residues 70−81 induced by binding to PG are shown (yellow arrows) (lower). Figure adapted from ref 67.

ologies are yielding new insight into the lipids that surround membrane proteins and will prove key for further structural and functional investigations.

Recent studies also suggest future directions, including the potential to "deorphanise" membrane proteins. "Deorphanizing" is an expression used typically with G-protein coupled receptors in which substrates are identified and consequently functions assigned.[68] Given that the majority of ABC transporters are of unknown function, the ability to identify substrates that copurify with ABC transporters has the potential to add important functional information. For example, the discovery of an endogenous lipid A substrate in complex with TmrAB, and its binding affinity linked to ATP turnover, suggested a role for TmrAB as a lipid flippase.[64] We anticipate that similar studies will aid in assigning roles to the many human ABC transporters for which there is currently no clearly ascribed function.

Since it is established that natural lipid bilayers are asymmetric and consist of membrane proteins with lipids in dynamic equilibrium, capturing these aspects of asymmetry and movement is of fundamental importance. In this regard, the ability to transmit membrane proteins from bicelles or nanodiscs,[69] in which the lipid environment can be regulated or preserved, as in native nanodiscs,[70] offers exciting opportunities for coupling with MS. Incorporation of isotopi-

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cally labeled lipids into these environments has the potential to capture the dynamics of lipid binding. Moreover, new developments in high-resolution MS of protein complexes,[71,72] when linked to the study of membrane complexes, will offer opportunities to couple important PTMs with lipid binding properties.

Taken together, knowledge of the depth of lipids surrounding a complex, the specificity and stoichiometry of lipid binding in isolated complexes, and the effects of lipid binding on protein stability have provided new insight into protein−lipid interactions. With new developments, designed to capture the dynamics and effects of PTMs, the role of MS is set to continue expanding into this fascinating and largely uncharted territory of membrane protein lipid interactions.

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Notes

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REFERENCES

(1) White, S. H. *Nature* **2009**, *459* (7245), 344−346.

(2) Nygaard, R.; Zou, Y.; Dror, R. O.; Mildorf, T. J.; Arlow, D. H.; Manglik, A.; Pan, A. C.; Liu, C. W.; Fung, J. J.; Bokoch, M. P.; Thian, F. S.; Kobilka, T. S.; Shaw, D. E.; Mueller, L.; Prosser, R. S.; Kobilka, B. K. *Cell* **2013**, *152* (3), 532−542.

(3) Colvin, M. T.; Andreas, L. B.; Chou, J. J.; Griffin, R. G. *Biochemistry* **2014**, *53* (38), 5987−5994.

(4) Nizsaloczki, E.; Csomos, I.; Nagy, P.; Fazekas, Z.; Goldman, C. K.; Waldmann, T. A.; Damjanovich, S.; Vamosi, G.; Matyus, L.; Bodnar, A. *ChemPhysChem* **2014**, *15* (18), 3969−3978.

(5) Chakraborty, H.; Chattopadhyay, A. *ACS Chem. Neurosci.* **2014**, *6* (1), 199−206.

(6) Kim, J.; Wu, S.; Tomasiak, T. M.; Mergel, C.; Winter, M. B.; Stiller, S. B.; Robles-Colmanares, Y.; Stroud, R. M.; Tampe, R.; Craik, C. S.; Cheng, Y. *Nature* **2014**, *517* (7534), 396−400.

(7) Wang, H.; Elferich, J.; Gouaux, E. *Nat. Struct Mol. Biol.* **2012**, *19* (2), 212−219.

(8) Whitelegge, J. P. *Anal. Chem.* **2013**, *85* (5), 2558−2568.

(9) Chung, K. Y.; Rasmussen, S. G.; Liu, T.; Li, S.; DeVree, B. T.; Chae, P. S.; Calinski, D.; Kobilka, B. K.; Woods, V. L., Jr.; Sunahara, R. K. *Nature* **2011**, *477* (7366), 611−615.

(10) Parker, C. H.; Morgan, C. R.; Rand, K. D.; Engen, J. R.; Jorgenson, J. W.; Stafford, D. W. *Biochemistry* **2014**, *53* (9), 1511−1520.

(11) Padayatti, P. S.; Wang, L.; Gupta, S.; Orban, T.; Sun, W.; Salom, D.; Jordan, S. R.; Palczewski, K.; Chance, M. R. *Mol. Cell Proteomics* **2013**, *12* (5), 1259−1271.

(12) Efremov, R. G.; Leitner, A.; Aebersold, R.; Raunser, S. *Nature* **2014**, *517* (7532), 39−43.

(13) Schmidt, C.; Zhou, M.; Marriott, H.; Morgner, N.; Politis, A.; Robinson, C. V. *Nat. Commun.* **2013**, *4*, 1985.

(14) Wyttenbach, T.; Pierson, N. A.; Clemmer, D. E.; Bowers, M. T. *Annu. Rev. Phys. Chem.* **2014**, *65*, 175−196.

(15) Bohrer, B. C.; Merenbloom, S. I.; Koeniger, S. L.; Hilderbrand, A. E.; Clemmer, D. E. *Annu. Rev. Anal Chem. (Palo Alto Calif)* **2008**, *1*, 293−327.

(16) Niu, S.; Rabuck, J. N.; Ruotolo, B. T. *Curr. Opin Chem. Biol.* **2013**, *17* (5), 809−817.

(17) Zhou, M.; Robinson, C. V. *Curr. Opin Struct Biol.* **2014**, *28C*, 122−130.

(18) Konijnenberg, A.; Yilmaz, D.; Ingolfsson, H. I.; Dimitrova, A.; Marrink, S. J.; Li, Z.; Venien-Bryan, C.; Sobott, F.; Kocer, A. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (48), 17170−17175.

(19) Wolynes, P. G. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92* (7), 2426−2427.

(20) Demmers, J. A.; Haverkamp, J.; Heck, A. J.; Koeppe, R. E., 2nd; Killian, J. A. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (7), 3189−3194.

(21) Demmers, J. A.; van Duijn, E.; Haverkamp, J.; Greathouse, D. V.; Koeppe, R. E., II; Heck, A. J.; Killian, J. A. *J. Biol. Chem.* **2001**, *276* (37), 34501−34508.

(22) Demmers, J. A.; van Dalen, A.; de Kruijff, B.; Heck, A. J.; Killian, J. A. *FEBS Lett.* **2003**, *541* (1−3), 28−32.

(23) Loo, R. R.; Dales, N.; Andrews, P. C. *Methods Mol. Biol.* **1996**, *61*, 141−160.

(24) Loo, R. R.; Dales, N.; Andrews, P. C. *Protein Sci.* **1994**, *3* (11), 1975−1983.

(25) Ilag, L. L.; Ubarretxena-Belandia, I.; Tate, C. G.; Robinson, C. V. *J. Am. Chem. Soc.* **2004**, *126* (44), 14362−14363.

(26) Kitagawa, N.; Mazon, H.; Heck, A. J.; Wilkens, S. *J. Biol. Chem.* **2008**, *283* (6), 3329−3337.

(27) Esteban, O.; Bernal, R. A.; Donohoe, M.; Videler, H.; Sharon, M.; Robinson, C. V.; Stock, D. *J. Biol. Chem.* **2008**, *283* (5), 2595−2603.

(28) Barrera, N. P.; Di Bartolo, N.; Booth, P. J.; Robinson, C. V. *Science* **2008**, *321* (5886), 243−246.

(29) Siuzdak, G.; Bothner, B. *Angew. Chem., Int. Ed. Engl.* **1995**, *34* (18), 2053−2055.

(30) Sharon, M.; Ilag, L. L.; Robinson, C. V. *J. Am. Chem. Soc.* **2007**, *129* (28), 8740−8746.

(31) Leon, I.; Millan, J.; Cocinero, E. J.; Lesarri, A.; Fernandez, J. A. *Angew. Chem., Int. Ed. Engl.* **2013**, *52* (30), 7772−7775.

(32) Fang, Y.; Bennett, A.; Liu, J. *Int. J. Mass Spectrom.* **2010**, *293* (1−3), 12−22.

(33) Fang, Y.; Bennett, A.; Liu, J. *Phys. Chem. Chem. Phys.* **2011**, *13* (4), 1466−1478.

(34) Barrera, N. P.; Isaacson, S. C.; Zhou, M.; Bavro, V. N.; Welch, A.; Schaedler, T. A.; Seeger, M. A.; Miguel, R. N.; Korkhov, V. M.; van Veen, H. W.; Venter, H.; Walmsley, A. R.; Tate, C. G.; Robinson, C. V. *Nat. Methods* **2009**, *6* (8), 585−587.

(35) Zhou, M.; Morgner, N.; Barrera, N. P.; Politis, A.; Isaacson, S. C.; Matak-Vinkovic, D.; Murata, T.; Bernal, R. A.; Stock, D.; Robinson, C. V. *Science* **2011**, *334* (6054), 380−385.

(36) Marcoux, J.; Wang, S. C.; Politis, A.; Reading, E.; Ma, J.; Biggin, P. C.; Zhou, M.; Tao, H.; Zhang, Q.; Chang, G.; Morgner, N.; Robinson, C. V. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (24), 9704−9709.

(37) Housden, N. G.; Hopper, J. T.; Lukoyanova, N.; Rodriguez-Larrea, D.; Wojdyla, J. A.; Klein, A.; Kaminska, R.; Bayley, H.; Saibil, H. R.; Robinson, C. V.; Kleanthous, C. *Science* **2013**, *340* (6140), 1570−1574.

(38) Barrera, N. P.; Zhou, M.; Robinson, C. V. *Trends Cell Biol.* **2013**, *23* (1), 1−8.

(39) Friemann, R.; Larsson, D. S.; Wang, Y.; van der Spoel, D. *J. Am. Chem. Soc.* **2009**, *131* (46), 16606−16607.

(40) Wang, Y.; Larsson, D. S.; van der Spoel, D. *Biochemistry* **2009**, *48* (5), 1006−1015.

(41) Patriksson, A.; Marklund, E.; van der Spoel, D. *Biochemistry* **2007**, *46* (4), 933−945.

(42) Borysik, A. J.; Robinson, C. V. *Phys. Chem. Chem. Phys.* **2012**, *14* (42), 14439−14449.

(43) Borysik, A. J.; Hewitt, D. J.; Robinson, C. V. *J. Am. Chem. Soc.* **2013**, *135* (16), 6078−6083.

(44) Han, L.; Hyung, S. J.; Mayers, J. J.; Ruotolo, B. T. *J. Am. Chem. Soc.* **2011**, *133* (29), 11358−11367.

DOI: 10.1021/jacs.5b00420
J. Am. Chem. Soc. 2015, 137, 5240−5247

(45) Bagal, D.; Kitova, E. N.; Liu, L.; El-Hawiet, A.; Schnier, P. D.; Klassen, J. S. *Anal. Chem.* **2009**, *81* (18), 7801−7806.
(46) Laganowsky, A.; Reading, E.; Hopper, J. T.; Robinson, C. V. *Nat. Protoc* **2013**, *8* (4), 639−651.
(47) Sobott, F.; Hernandez, H.; McCammon, M. G.; Tito, M. A.; Robinson, C. V. *Anal. Chem.* **2002**, *74* (6), 1402−1407.
(48) Gutmann, D. A.; Mizohata, E.; Newstead, S.; Ferrandon, S.; Postis, V.; Xia, X.; Henderson, P. J.; van Veen, H. W.; Byrne, B. *Protein Sci.* **2007**, *16* (7), 1422−1428.
(49) Reading, E.; Liko, I.; Allison, T. M.; Benesch, J. L.; Laganowsky, A.; Robinson, C. V. *Angew. Chem., Int. Ed. Engl.* **2015**, In press.
(50) Lemieux, M. J.; Reithmeier, R. A.; Wang, D. N. *J. Struct Biol.* **2002**, *137* (3), 322−332.
(51) Mehmood, S.; Marcoux, J.; Hopper, J. T.; Allison, T. M.; Liko, I.; Borysik, A. J.; Robinson, C. V. *J. Am. Chem. Soc.* **2014**, *136* (49), 17010−17012.
(52) Leney, A. C.; McMorran, L. M.; Radford, S. E.; Ashcroft, A. E. *Anal. Chem.* **2012**, *84* (22), 9841−9847.
(53) Calabrese, A. N.; Watkinson, T. G.; Henderson, P. J.; Radford, S. E.; Ashcroft, A. E. *Anal. Chem.* **2015**, *87* (2), 1118−1126.
(54) Lee, A. G. *Biochem. Soc. Trans.* **2011**, *39* (3), 761−766.
(55) Yeagle, P. L. *Biochim. Biophys. Acta* **2014**, *1838* (6), 1548−1559.
(56) King, G.; Sharom, F. J. *Crit Rev. Biochem. Mol. Biol.* **2012**, *47* (1), 75−95.
(57) Murata, T.; Yamato, I.; Kakinuma, Y.; Leslie, A. G.; Walker, J. E. *Science* **2005**, *308* (5722), 654−659.
(58) Hansen, S. B.; Tao, X.; MacKinnon, R. *Nature* **2011**, *477* (7365), 495−498.
(59) Arias-Cartin, R.; Grimaldi, S.; Pommier, J.; Lanciano, P.; Schaefer, C.; Arnoux, P.; Giordano, G.; Guigliarelli, B.; Magalon, A. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (19), 7781−7786.
(60) Hopper, J. T.; Robinson, C. V. *Angew. Chem., Int. Ed. Engl.* **2014**, *53* (51), 14002−14015.
(61) Watt, I. N.; Montgomery, M. G.; Runswick, M. J.; Leslie, A. G.; Walker, J. E. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (39), 16823−16827.
(62) Lee, A. G. *Mol. Biosyst* **2005**, *1* (3), 203−212.
(63) Velamakanni, S.; Lau, C. H.; Gutmann, D. A.; Venter, H.; Barrera, N. P.; Seeger, M. A.; Woebking, B.; Matak-Vinkovic, D.; Balakrishnan, L.; Yao, Y.; U, E. C.; Shilling, R. A.; Robinson, C. V.; Thorn, P.; van Veen, H. W. *PLoS One* **2009**, *4* (7), e6137.
(64) Bechara, C.; Nöll, A.; Morgner, N.; Degiacomi, M. T.; Tampe, R.; Robinson, C. V. *Nat. Chem.* **2015**, *7* (3), 255−262.
(65) Zutz, A.; Hoffmann, J.; Hellmich, U. A.; Glaubitz, C.; Ludwig, B.; Brutschy, B.; Tampe, R. *J. Biol. Chem.* **2011**, *286* (9), 7104−7115.
(66) Sharom, F. J. *IUBMB Life* **2011**, *63* (9), 736−746.
(67) Laganowsky, A.; Reading, E.; Allison, T. M.; Ulmschneider, M. B.; Degiacomi, M. T.; Baldwin, A. J.; Robinson, C. V. *Nature* **2014**, *510* (7503), 172−175.
(68) Civelli, O.; Reinscheid, R. K.; Zhang, Y.; Wang, Z.; Fredriksson, R.; Schioth, H. B. *Annu. Rev. Pharmacol Toxicol* **2013**, *53*, 127−146.
(69) Hopper, J. T.; Yu, Y. T.; Li, D.; Raymond, A.; Bostock, M.; Liko, I.; Mikhailov, V.; Laganowsky, A.; Benesch, J. L.; Caffrey, M.; Nietlispach, D.; Robinson, C. V. *Nat. Methods* **2013**, *10* (12), 1206−1208.
(70) Dorr, J. M.; Koorengevel, M. C.; Schafer, M.; Prokofyev, A. V.; Scheidelaar, S.; van der Cruijsen, E. A.; Dafforn, T. R.; Baldus, M.; Killian, J. A. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (52), 18607−18612.
(71) Rose, R. J.; Damoc, E.; Denisov, E.; Makarov, A.; Heck, A. J. *Nat. Methods* **2012**, *9* (11), 1084−1086.
(72) Li, H.; Wongkongkathep, P.; Van Orden, S. L.; Ogorzalek Loo, R. R.; Loo, J. A. *J. Am. Soc. Mass Spectrom.* **2014**, *25* (12), 2060−2068.

DOI: 10.1021/jacs.5b00420
J. Am. Chem. Soc. 2015, 137, 5240−5247