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Published in:
Angewandte Chemie - International Edition

DOI:
10.1002/anie.201914411

Publication date:
2020

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Bolla, J. R., Corey, R. A., Sahin, C., Gault, J., Hummer, A., Hopper, J. T. S., Lane, D. P., Drew, D., Allison, T. M., Stansfeld, P. J., Robinson, C. V., & Landreh, M. (2020). A Mass-Spectrometry-Based Approach to Distinguish Annular and Specific Lipid Binding to Membrane Proteins. Angewandte Chemie - International Edition, 59(9), 3523-3528. https://doi.org/10.1002/anie.201914411
A Mass-Spectrometry-Based Approach to Distinguish Annular and Specific Lipid Binding to Membrane Proteins

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Abstract: Membrane proteins engage in a variety of contacts with their surrounding lipids, but distinguishing between specifically bound lipids, and non-specific, annular interactions is a challenging problem. Applying native mass spectrometry to three membrane protein complexes with different lipid-binding properties, we explore the ability of detergents to compete with lipids bound in different environments. We show that lipids in annular positions on the presenilin homologue protease are subject to constant exchange with detergent. By contrast, detergent-resistant lipids bound at the dimer interface in the leucine transporter show decreased $k_{\text{off}}$ rates in molecular dynamics simulations. Turning to the lipid flippase MurJ, we find that addition of the natural substrate lipid-II results in the formation of a 1:1 protein–lipid complex, where the lipid cannot be displaced by detergent from the highly protected active site. In summary, we distinguish annular from non-annular lipids based on their exchange rates in solution.

Membrane proteins rely on the lipid environment of the membrane to shape their structure and function, yet the nature of the underlying lipid interactions can be highly diverse. Annular lipids form non-specific contacts with the protein and exchange rapidly with the bulk lipids in the membrane. The function of annular lipids is to provide the amphiphilic environment required to keep the protein in a folded state, while non-annular lipids have more defined functional roles, such as stabilization of oligomeric assemblies or binding to regulatory sites. Due to the challenge of experimentally studying these proteins in the membrane, they are often solubilized by incubation with an excess amount of detergent in solution. This process breaks up the membrane and replaces protein–lipid interactions with protein–detergent ones. Interestingly, some detergent-solubilized proteins retain bound lipids, which are sometimes observed in the electron density maps of X-ray crystallography or cryo-electron microscopy experiments. These co-purified species are often specific lipids preferentially bound at non-annular sites and provide functionally important interactions.

Here we ask 1) whether we can observe the exchange of annular lipids for detergent molecules, and 2) whether non-annular lipids would exhibit different exchange properties. To answer these questions, we turned to native mass spectrometry (nMS), which enables the study of lipid binding to membrane proteins under a variety of solution conditions. After release from the detergent micelle inside the mass spectrometer, it is possible to detect intact protein–lipid complexes and to measure their binding stoichiometry and stability in the gas phase.

We can follow lipid binding by adding lipids to detergent-solubilized membrane proteins and measuring the molecular weight of the resulting complexes with nMS. We therefore hypothesized that it should also be possible to detect lipid-to-detergent exchange by measuring the fraction of bound exogenous lipids as a function of detergent concentration (Figure 1a). We selected the 33 kDa presenilin homologue PSH from Methanoculleus marisnigri, because it is well...
established that it can be extracted from the membrane as a lipid-free monomer and its activity is restored in detergent micelles with the addition of *Escherichia coli* lipids.[12,13] The addition of *E. coli* polar lipid extract to lipid-free PSH in 0.2% nonylglucoside (NG) and 0.03% dimethyl-dodecylamine N-oxide (LDAO) resulted in a nMS spectrum with additional peaks for each charge state. The detergent conditions used in this study are summarized in Table S1 in the Supporting Information. The mass difference between the peaks corresponded to binding of up to three lipids per protein (Figure 1b). Increasing the concentration of NG in a stepwise fashion from 0.2% to 0.5% led to a gradual removal of lipid adduct peaks, in line with the delipidating properties of NG[14] (Figure 1b). Together these results show that it is possible to follow lipid exchange with detergent in a concentration-dependent manner. Since we added all three of the main *E. coli* lipids simultaneously, phosphatidylethanolamines (PE), phosphatidylglycerols (PG), and cardiolipins (CDL), we could observe that increasing the concentration of NG induces stepwise removal of all three lipids, indicating no difference in the extent of removal of each lipid with respect to detergent concentration; the different lipids were displaced at comparable rates. We therefore conclude that there is no marked preference for binding of a particular class of lipid to PSH (Figure 1b and Supporting Information, Figure S1). The competition, observed experimentally for PSH, between lipids and detergent molecules is consistent with the annular lipid interactions predicted by molecular dynamics (MD) simulations of membrane proteins in mixed lipid–detergent micelles.[16] Lipids added to solubilized PSH are therefore in exchange with the detergent when binding to the protein, and become more likely to be replaced at increasing detergent concentrations, as directly seen by nMS (Figure 1c).

The second question is whether the exchange between bound lipids and detergent molecules could be used more generally as an indicator for non-annular, specific lipid interactions. To investigate whether we can distinguish lipids based on their exchange rates, we selected the leucine transporter LeuT from *Aquifex aeolicus*, which can be purified as a dimer with bound CDL and PG.[11] Incubation for 16 hours in 2% NG was found to remove the co-purified lipids and release monomeric protein as shown previously (Figure 2a).[11] These findings suggest that the lipids required for dimerization are retained when the protein is solubilized in detergent, and can only be removed by prolonged incubation in particularly harsh detergent conditions (Figure 2b).

To investigate this possibility, we performed coarse-grained molecular dynamics (CG-MD) simulations of LeuT in a mixed lipid bilayer and extracted the residency times and $k_{off}$ values (based on reference [15] for CDL binding to the protein). Three distinct sites on LeuT were defined based on previously generated free energy landscapes of CDL–LeuT...
interaction,\textsuperscript{[16]} including a site with very little observed specificity (R11/I441). For simplicity, we chose to represent each site with a pair of flanking residues (Figure 2c and Supporting Information, Figure S2). CDL bound to the R11/I441 sites remote from the dimer interface displayed very short residency times and $k_{\text{off}}$ rates greater than 70 m\(^{-1}\)s\(^{-1}\). CDL interactions at the R435/R446 site exhibited slower $k_{\text{off}}$ rates of 31–34 m\(^{-1}\)s\(^{-1}\), possibly due to the availability of two arginine sidechains for interactions with the negatively charged phosphate head-groups. However, CDL bound at the site composed of R88 and K376 across the dimer interface showed markedly higher residency times with some lipids bound for as long as 800 to 1000 ns, and $k_{\text{off}}$ rates of less than 10 m\(^{-1}\)s\(^{-1}\). As a result, the interfacial binding sites were occupied almost constantly over the course of our simulation. These findings suggest that the CDL bound to specific binding sites formed by the dimer interface exhibit a high resistance to the detergent competition. Lipids that detach more frequently, typically not interfacial lipids, have a higher probability of being displaced from the protein by detergent.

Having established that annular and non-annular lipids can differ in their exchange with detergent, we asked whether we can use solution competition to identify lipid interactions involving non-annular binding sites. We selected the 57 kDa \textit{E. coli} lipid flippase MurJ, a monomeric 14-helix integral membrane protein. MurJ catalyzes the transport of lipid-II, an essential precursor for the synthesis of cell wall peptidoglycans in bacteria, making it an important target for anti-
[17] The structural basis for MurJ flippase activity is only partially understood, primarily because the large, flexible lipid-II substrate is not resolved in crystal structures. We have previously shown that substrate binding to MurJ is inhibited by CDL, which blocks the lipid-II binding site. [19] nMS analysis of MurJ extracted from E. coli revealed a sub-population with a single co-purified CDL that is retained under a variety of detergent conditions (Supporting Information, Figure S3). This led us to speculate whether CDL or lipid-II, upon binding becomes isolated from the surrounding lipid or detergent environment. To investigate this question, we carried out analogous experiments to those performed for LeuT and PSH. We added 16:0–18:1 PE (POPE), the most abundant E. coli lipid and not a MurJ substrate, to the protein in 0.05% LDAO. nMS shows binding of multiple POPE molecules per protein (Figure 3a). The concentration of NG was then increased in a stepwise fashion and the effect on lipid binding monitored. We find that NG effectively competes with POPE for binding to the protein, reducing the number and intensity of lipid adducts in a concentration-dependent manner (Figure 3a). Using POPE binding to identify the most stringent competition...
conditions, we identified 0.5% octylglucoside (OG) as the most efficient detergent to disrupt lipid binding to MurJ (Supporting Information, Figure S4). The observation that in this case, OG removes bound lipids more readily than NG suggests that delipidation abilities of different detergents may vary between protein systems.

Next, we added the natural substrate lipid-II in 2-fold excess to MurJ in 0.05% LDAO. From the mass spectrum we observed apo protein and protein with lipid-II adducts, the dominant species being assigned to MurJ:(lipid-II)2 and MurJ:(lipid-II)3 (Figure 3b). Stripping LDAO from a desolubilized sample of MurJ isolated from the membrane.

The native accessibility of residues are completely inaccessible in the inward-closed state, we observed sporadic contacts between the acyl chains of UDP and all three arginine residues, suggesting that the active site is highly protected but remains occasionally accessible to the substrate. Strikingly, all three key residues are completely inaccessible in the inward-closed conformation, showing that the active site can be completely isolated from the membrane.

Together, these MS and MD data show therefore that while several lipids can readily bind to MurJ, only one lipid-II adduct does not readily exchange with the detergent, indicative of binding with a slower $k_{off}$ rate than the other lipids. Since lipid-II is the natural substrate of MurJ, we explored the accessibility of the active site in MD simulations using a derivative of lipid-II and found that a substrate can enter the active site most readily in the inward-facing conformation partially isolated from the surrounding membrane. Excess lipid-II, as well as other non-specifically bound E. coli lipids, can be exchanged with detergent, suggesting binding in annular positions (Figure 3c).

In summary, we have demonstrated a simple MS-based strategy to distinguish annular from non-annular lipids based on their ability to exchange with detergent in solution. For PSHI, we showed that annular lipids could be exchanged readily for detergent with no distinction between the various lipids tested. Supporting our observations with MD simulations, we then showed that in LeuT non-specifically bound lipids exchange more rapidly with the surrounding detergent than interfacial CDL, which exhibits slow $k_{off}$ rates, and are less likely to exchange with detergent. Using this detergent-competition assay, we were able to distinguish annular lipids from a single lipid-II molecule bound to MurJ when the active site is transiently accessible. We believe that our approach may provide valuable insights into the distinction of annular and non-annular lipids that modulate the structure and function of membrane proteins.

**Acknowledgements**

The authors would like to extend special thanks to Dr. Shenzhu Wu and Prof. Yigong Shi, Tsinghua University, PR China, for kindly providing the PSHI protein, and Dr. Kallof Gupta, Yale University, USA, for the LeuT MS data. M.L. acknowledges technical support from MS Vision, NL, and is supported by an Ingvar Carlson Award from the Swedish Foundation for Strategic Research, a KI faculty-funded Career Position, a VR starting grant, and a KI-StratNeuro starting grant. D.P.L. is supported by VR grant 2013_08807. C.V.R. is funded by a Wellcome Trust Investigator Award (104633/Z/14/Z), an ERC Advanced Grant ENABLE (641317), and an MRC Programme Grant (MR/N020413/1). P.J.S. and R.A.C. were funded by Wellcome [208361/Z/17/Z]. Research in P.J.S.’s lab is funded by the MRC (MR/S009213/1) and BBSRC (BB/P01948X/1, BB/R002517/1, BB/S003339/1). This project made use of time on ARCHER granted via the UK High-End Computing Consortium for Biomolecular Simulation HECBioSim (http://hecbiosim.ac.uk), supported by EPSRC (grant no. EP/R029407/1) and Athena at HPC Midlands +, which is supported by the EPSRC (EP/P020323/1), and the University of Warwick Scientific Computing Research Technology Platform. C.V.R. and J.G. perform consultancy services for OMass Therapeutics Ltd and J.T.S. is an employee of that company. J.G. is a Junior Research Fellow at The Queen’s College, Oxford.
Conflict of interest
The authors declare no conflict of interest.

Keywords: lipid binding · membrane protein structure · molecular dynamics · native mass spectrometry

How to cite: Angew. Chem. Int. Ed. 2020, 59, 3523–3528
Angew. Chem. 2020, 132, 3551–3556

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Manuscript received: November 12, 2019
Revised manuscript received: December 29, 2019
Accepted manuscript online: December 30, 2019
Version of record online: January 29, 2020