Membrane Proteins

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Interrogating Membrane Protein Conformational Dynamics within Native Lipid Compositions

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Abstract: *The interplay between membrane proteins and the lipids of the membrane is important for cellular function, however, tools enabling the interrogation of protein dynamics within native lipid environments are scarce and often invasive. We show that the styrene–maleic acid lipid particle (SMALP) technology can be coupled with hydrogen–deuterium exchange mass spectrometry (HDX-MS) to investigate membrane protein conformational dynamics within native lipid bilayers. We demonstrate changes in accessibility and dynamics of the rhomboid protease GlpG, captured within three different native lipid compositions, and identify protein regions sensitive to changes in the native lipid environment. Our results illuminate the value of this approach for distinguishing the putative role(s) of the native lipid composition in modulating membrane protein conformational dynamics.*

Cell membranes are highly complex and dynamic organizations of lipids and membrane proteins that are responsible for many vital cellular functions. Previous work has demonstrated that lipid–protein interactions influence membrane protein folding, structure, dynamics, and function.[1] However, the influence of the actual native lipid composition encountered in the membrane has largely eluded investigation. It is imperative, therefore, to be able to explore the interplay between native lipid environments and membrane proteins to fully understand cellular membrane protein function.

To investigate their structure and dynamics, membrane proteins are typically solubilized and purified from their membrane using detergent micelles. This approach has been successful for studying membrane proteins, however, these systems lack the context of their native lipid constituents. Recently styrene–maleic acid lipid particle (SMALP) technology has been developed, which uses SMA to directly solubilize membrane proteins from their native membranes into nanodiscs (Figure 1a and Figure S1). These nanodiscs have been termed "native nanodiscs" to distinguish them from reconstituted nanodisc technologies.[2] Excitingly, this enables membrane proteins to be captured in nanodiscs containing a lipid make-up akin to its native membrane. This technology has been successful for studying a variety of bacterial and human membrane proteins.[2] Herein, we establish that the SMALP technology can be coupled with hydrogen–deuterium exchange mass spectrometry (HDX-MS) to afford a generic method for studying membrane protein conformational dynamics within native lipid compositions.

HDX-MS measures the rate of exchange of deuterium with backbone amide hydrogen atoms upon incubation in D_2O-containing buffer, which depends on solvent accessibility and hydrogen bonding. HDX from solvent-exposed protein regions likely dominates at the earliest times of incubation, providing information on protein structure. Over time, slow (local and global) structural transitions, concomitant with protein motions, result in transient exchange events of otherwise protected amide hydrogens, thereby providing a direct measurement of protein conformational dynamics.[3] This method differs from other approaches in that it enables dynamic structural information to be captured rapidly with small amounts of material, does not require chemical modification or sequence alterations, and can be used to analyze complex mixtures. These advantages have contributed to its wide adoption in both the academic and pharmaceutical sectors,[4] nevertheless, its application to membrane proteins is still in its infancy. Although HDX-MS of membrane proteins within reconstituted nanodiscs has been successful,[5] it has not yet been exploited to study membrane proteins within native nanodiscs (which have a native lipid composition).

We used the *Escherichia coli* rhomboid protease GlpG as a model system since membrane immersion of this protein has been observed to be essential for functional specificity.[6] Rhomboid proteases are a nearly ubiquitous family of intramembrane serine proteases that cleave peptide bonds within the lipid bilayer, and they are implicated in a number of diseases.[7] GlpG consists of a six α-helix transmembrane (TM) domain, which contains the catalytic dyad, and an α-helical/β-structured cytoplasmic domain (CytD; Figure 1a). High-resolution structures have been solved for both domains separately but not for the complete GlpG protein;[8] with structural information on the functionally important linker region (residues 68–90, Ln) currently unavailable.[9]

We aimed to capture GlpG in native nanodiscs consisting of different native lipid compositions by using cell lines with subtly different lipid compositions [BL21(DE3) (BL) and C43(DE3) (C)],[10] and by altering the temperature upon induction (37 or 16 °C) to further modulate the lipid

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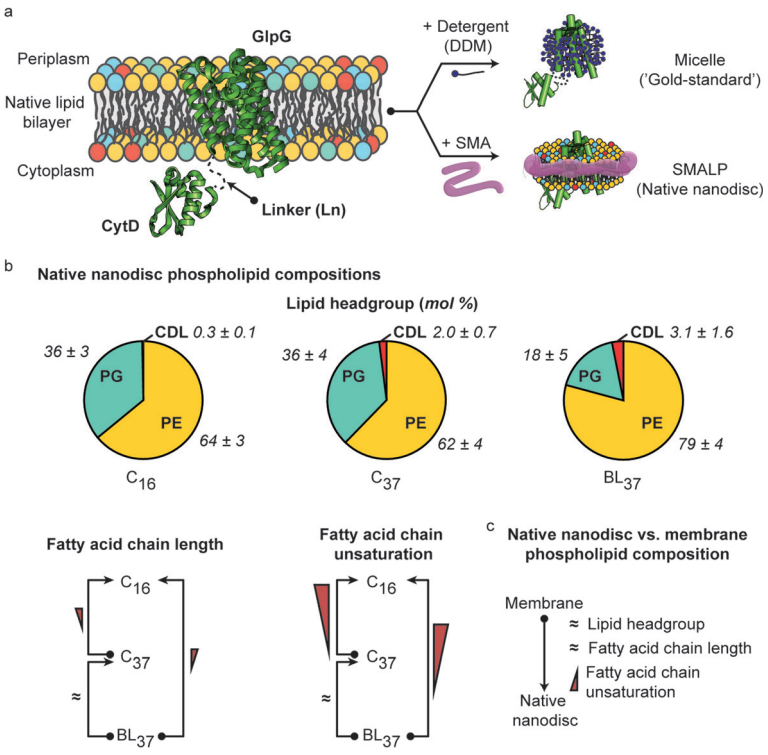

Figure 1. GlpG within native nanodiscs (SMALPs) of varying native lipid composition. a) The GlpG membrane domain (PDB ID: 2XTV) is connected to its cytoplasmic N-terminal domain (CytD, PDB ID: 2LEP) by a linker region (residues 68–90, Ln), for which there is currently has no structural information available. As an alternative to solubilization with *n*-dodecyl β-D-maltoside (DDM) detergent to form a protein–detergent micelle complex, GlpG is solubilized from its native bilayer with the SMA polymer to form SMALPs (native nanodiscs), which contain a native lipid bilayer. b) Lipidomics analysis of native nanodiscs containing GlpG. Phospholipid head group compositions of the three native nanodisc systems (C_{16}, C_{37}, and BL_{37}) and the differences in chain length and chain saturation. c) Differences in phospholipid head group composition, chain length, and chain saturation between native cell membranes and native nanodiscs. Black arrows represent the direction of comparison and the red ramps represent the direction and degree (small or large) of change. The phospholipid head group, and chain length and saturation compositions, for both the native nanodiscs and the native cell membrane are reported in Table S2 and Figure S4.

content.[11] The three native nanodiscs attained are termed BL_{37}, C_{37}, and C_{16}. We found that GlpG was pure, folded, thermally stable, and functional within these homogenous native nanodiscs (Figure S2 and Table S1 in the Supporting Information). We also measured the inorganic phosphate content of the purified nanodiscs to confirm and quantify the presence of phospholipids (see the Methods section in the Supporting Information), which revealed approximately 140 phospholipids/GlpG monomer. A GlpG crystal structure within a lipid environment revealed that 14 fully or partially ordered lipid molecules form a partial annulus around the protein,[12] therefore, the native lipid encapsulated by the SMA polymer is likely sufficient to capture a complete lipid annulus around GlpG.

Using lipidomics, we measured the composition and relative abundances of the lipids within the GlpG native nanodiscs (Figure 1b). We identified around 50 individual lipids from three major classes: phosphatidylglycerols (PG), phosphatidylethanolamines (PE), and cardiolipins (CDL; Figure S3 and Table S2), which is consistent with the known composition of *Escherichia coli* cell membranes.[11a] Furthermore, our analysis revealed that BL_{37} native nanodiscs had a relatively decreased PG content and increased PE and CDL content, compared to C_{37} and C_{16} native nanodiscs. Whilst the PE and PG content of C_{37} and C_{16} were similar, there was a relative decrease in CDLs within C_{16} compared to C_{37}. In all three cases, there were no differences observed in the lipid headgroup composition for membranes compared to their native nanodiscs (Figure 1c), thus highlighting their similar lipid environments.

Next, we used tandem MS to fragment the intact lipids, to identify the fatty acid chains attached to the different headgroups (Figure 1b and Figure S4). Overall, the saturations and chain lengths were similar between C_{37} and BL_{37}, however increased chain unsaturation was observed for C_{16}. Small changes in chain length were also observed at the lower temperature; with a modest increase in abundance of longer-chain-length lipids. These observations are in line with previous reports that showed that *Escherichia coli* bacteria increase the chain unsaturation and proportion of *cis*-vaccenoyl chains (18:1c11), with a decrease in palmitoyl chains (16:0), in response to cold-shock.[11] An increase in chain unsaturation leads to looser lipid packing, thereby increasing membrane fluidity at lower temperatures.

We then established a HDX-MS approach compatible with the SMALP technology. We optimized the quenching and digestion conditions, as well as the overall workflow, using the C_{37} native nanodiscs (Figure S5 and Table S3). By applying the same conditions to the other two native nanodiscs (C_{16} and BL_{37}), we could achieve peptide coverage sufficient for investigating the effect that alterations in native lipid composition have on GlpG (Figure 2a and Figure S6).

By observing the relative fractional uptake of deuterium by GlpG across all the native lipid compositions, we could make some general inferences (Figure 2b and Figure S7–8). Peptides within TM2-6 of GlpG were relatively protected from HDX, as demonstrated by a low relative fractional uptake, probably because these regions are buried within the hydrophobic core of the membrane. The CytD and Ln regions of GlpG displayed much higher relative fractional uptake of deuterium in general. Surprisingly, the TM region linked to the cytoplasmic domain of GlpG (TM1, peptide 94–108) was relatively unprotected from HDX and showed dynamic behavior.

We then directly compared relative deuterium uptake between the three native lipid compositions (Figure 3). This enabled us to pinpoint regions of GlpG that are influenced by subtle alterations in their lipid environment—informed by changes in either their protection against HDX and/or HDX dynamics. We found that GlpG possessed similar HDX

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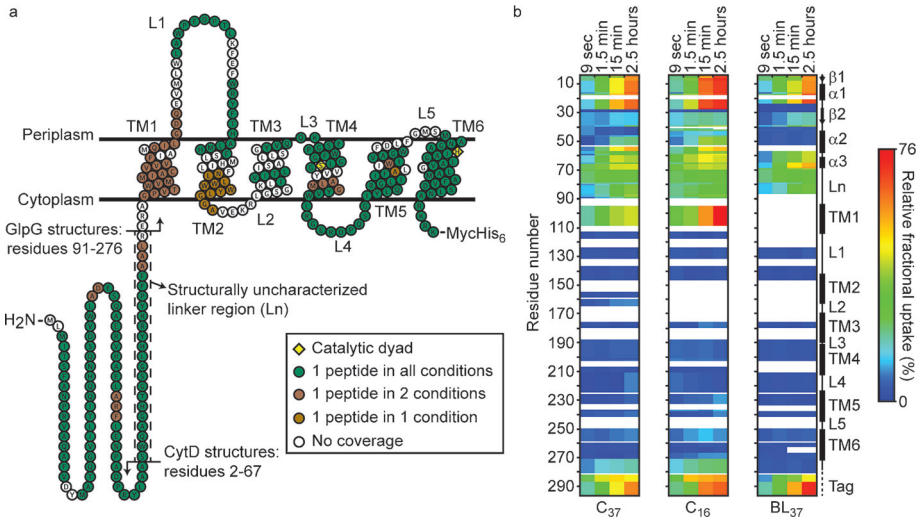

Figure 2. HDX-MS of GlpG in native nanodiscs (SMALPs). a) GlpG peptide coverage across the three native nanodisc compositions. Details on the high-resolution structural information available for GlpG are included. b) Heat maps representing the relative fractional uptake of deuterium for peptides of GlpG in the three native nanodiscs. The secondary structure of GlpG is shown on the right (black); β-strands (β) are depicted as arrows, loop (L) regions and linker (Ln) regions as lines, the purification tag as dotted lines, and α-helices (α) and transmembrane helices (TM) as bars. The degree of relative fractional uptake of deuterium at each incubation time (from left to right: 9 s, 90 s, 900 s, and 9000 s) is displayed according to the color code shown. Uncolored regions indicate areas with no peptide coverage.

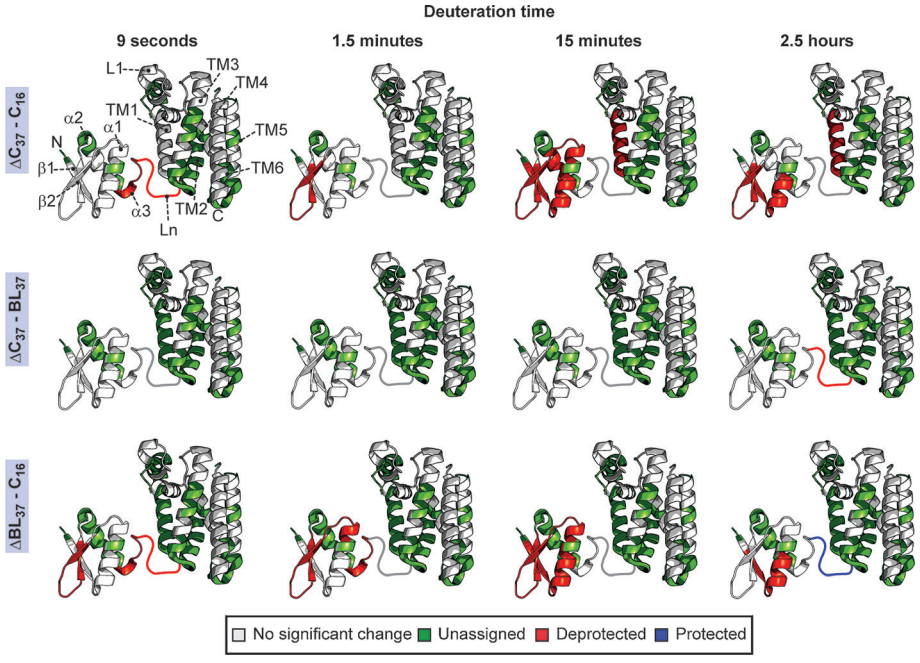

Figure 3. Differences in relative deuterium uptake (ΔHDX) by GlpG in different native nanodiscs. HDX behavior is mapped onto the GlpG (PDB ID: 2XTV) and CytD (PDB ID: 2LEP) crystal structures for each deuteration incubation time. Red and blue colored regions indicate GlpG segments containing a peptide that becomes HDX deprotected or protected, respectively; gray regions indicate that no significant ΔHDX is observed for any peptide, and green indicates regions of GlpG where peptides were not obtained for both conditions. A significant change in ΔHDX was determined as more than 0.9 Da (99% confidence interval). The ΔHDX data is presented in a Woods plot in Figure S7.

behavior in the C_{37} and BL_{37} nanodiscs despite significant differences in PE and PG compositions (PE/PG ratio: $C_{37} = 1.7 \pm 0.2$, $BL_{37} = 4.4 \pm 1.3$); however, at the maximum deuteration time (2.5 hours), the Ln region (peptide 68–78) was deprotected from HDX within the BL_{37} nanodisc. The most striking differences in HDX in GlpG were found for the C_{16} nanodisc environment compared to both the BL_{37} and C_{37} nanodiscs. Here, we observed peptides within the CytD, Ln, and TM1 regions that were typically deprotected within the C_{16} environment. This is likely due the C_{16} nanodisc possessing a more fluid bilayer, which facilitates GlpG structural fluctuations.

Overall, the HDX-MS analysis proved powerful in revealing regions that are strongly influenced by alterations in the native lipid environment—the CtyD, Ln, and TM1 regions. Interestingly, the TM1 region has been found to be important for structural stability[13] and a preferred interaction site (close to TM1/TM3) for the rhomboid protease Spitz substrate.[14] The CtyD domain can undergo domain swapping in isolation from the GlpG domain,[15] but currently the functional importance of this region is unknown. Interestingly, cytosolic extensions in other rhomboid proteases have been found to be important for modulating substrate gating.[16] The Ln region has been shown to be important for maintaining maximum GlpG activity in some assays.[9a] Due to its position within the protein (connecting the GlpG and CtyD domains) it is likely to be in direct or close contact with the membrane. We therefore propose that this lipid-sensitive region may play a role in the function of GlpG through interactions with the membrane. Changes in the PE/PG ratio between C_{37} and BL_{37} did not seem to affect HDX significantly, whereas changes in chain length and saturation between C_{37} and C_{16} did. This may be related to previous evidence from detergent micelle and bicelle systems that hydropho-

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bic mismatch could exert an inhibitory effect on GlpG activity.[9b]

The potential of this method in examining lipid, ligand, and drug interactions with membrane proteins, in well-defined native lipid environments, is an exciting prospect that we anticipate will have a significant impact on membrane protein structural biology, as well as on drug discovery.

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Conflict of interest

The authors declare no conflict of interest.

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