

Engineering cardiolipin binding to an artificial membrane protein reveals determinants for lipid-mediated stabilization

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Cardiolipin is known to play an **important** role in modulating the assembly and function of membrane proteins in bacterial and mitochondrial membranes. Here, authors **convincingly** define the molecular determinants of cardiolipin binding on de novo-designed and native membrane proteins combining the coarse-grained molecular dynamics simulation with the state-of-the-art experimental approaches such as native mass spectrometry and cryogenic electron microscopy. The major findings in this study, which are the identification of degenerate cardiolipin binding motifs, the characterization of their dynamic features, and the role in membrane protein stability and activity, will provide much needed insight into the still poorly understood nature of protein-cardiolipin interactions.

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Abstract

Integral membrane proteins carry out essential functions in the cell, and their activities are often modulated by specific protein-lipid interactions in the membrane. Here, we elucidate the intricate role of cardiolipin (CDL), a regulatory lipid, as a stabilizer of membrane proteins and their complexes. Using the *in silico*-designed model protein TMHC4_R (ROCKET) as a scaffold, we employ a combination of molecular dynamics simulations and native mass

spectrometry to explore the protein features that facilitate preferential lipid interactions and mediate stabilization. We find that the spatial arrangement of positively charged residues as well as local conformational flexibility are factors that distinguish stabilizing from non-stabilizing CDL interactions. However, we also find that even in this controlled, artificial system, a clear-cut distinction between binding and stabilization is difficult to attain, revealing that overlapping lipid contacts can partially compensate for the effects of binding site mutations. Extending our insights to naturally occurring proteins, we identify a stabilizing CDL site within the *E. coli* rhomboid intramembrane protease GlpG and uncover its regulatory influence on enzyme substrate preference. In this work, we establish a framework for engineering functional lipid interactions, paving the way for the design of proteins with membrane-specific properties or functions.

Introduction

Biological membranes, which are vital for cellular life, provide a specific and highly adaptable lipid environment for membrane proteins that govern numerous cellular functions (1). The exact roles that the different membrane lipids play in the regulation of membrane proteins often go unacknowledged, as their highly dynamic interactions challenge conventional analytical methods. Despite these obstacles, evidence has consistently highlighted the crucial role of lipids (2), for example as allosteric regulators (3), facilitating protein oligomerization (4), or locally affecting the properties of the membrane (5). The simplest form of lipid-mediated regulation is the stabilization of specific protein conformations (6), resulting in the observation of individual lipid molecules in high-resolution structures (7). These “structural” lipids often display increased residence times at their binding sites which distinguish them from non-regulatory, “annular” lipids (8).

Cardiolipin (CDL) is a prime example of a lipid with regulatory activity for both bacterial and mitochondrial membrane proteins (9). Due to its unique structure, comprised of two phosphate groups which both potentially carry a negative charge, and four acyl chains, CDL mediates the assembly of membrane protein oligomers, for example in the respiratory chain supercomplexes (10). The double phosphate groups can create strongly attractive electrostatic interactions with basic side chains, which makes CDL an ideal model lipid to understand interactions, but it also exhibits more specific patterns. Of note, both the head groups and all four acyl chains are thought to be important components of supercomplex stabilization (11). Similarly, CDL plays an essential role in the dimerization of the Na^+/H^+ antiporter NhaA, which increases the exchanger activity to protect the bacteria from osmotic stress (12, 13). In addition, CDL can affect the activity of other membrane proteins such as ADP/ATP carrier Aac2 and magnesium transporter MgtA by acting as an allosteric regulator (14, 15). Therefore, sites displaying preferential CDL binding may indicate lipid-activated regulatory mechanisms. To address this possibility, we have previously used coarse-grained molecular dynamics (CG-MD) simulations to map CDL binding sites on *E. coli* inner membrane proteins with published structures, identifying specific amino acids and binding site geometries that mediate preferential interactions with CDL (16). Although such CDL “fingerprints” are found in a wide range of proteins with different activities, they stop short of clarifying the functional role of lipids at these sites, with predictions of their functionality remaining largely speculative. Addressing this knowledge gap requires monitoring both the molecular interactions as well as the structure or stability of membrane protein complexes. For instance, thermal-shift assays provide data on lipid binding and associated changes in protein stability, which may indicate a functionally or structurally important lipid interaction (17). Moreover, native mass spectrometry (nMS) has gained traction for membrane protein analysis, revealing the influence of lipids on oligomerization (18), binding affinities (19), and conformational stability (20). Monitoring mass shifts captures individual lipid interactions

across multiple protein populations, while gas-phase dissociation provides insight into lipid stabilization. nMS thus captures key features of regulatory lipid interactions, and is especially powerful when coupled with MD which provides insight at the atomistic level (21 [↗](#)).

Being able to connect individual lipid binding events to the stability of a protein complex is a crucial step towards predicting functionally important CDL interactions. We reasoned that a combined MD and nMS strategy may reveal basic requirements for CDL-mediated stabilization. However, the sequence and structures of membrane proteins are evolutionarily entrenched with the lipid composition of their surrounding membrane. To reduce the system to first principles, we turned to TransMembrane Helical Core Tetramer_Rocket-shaped (TMHC4_R, hereafter referred to as ROCKET), an artificial membrane protein tetramer whose sequence was derived from Rosetta Monte Carlo calculations (22 [↗](#)). ROCKET includes a generic lipid-water interface composed of a ring of aromatic residues and a ring of positively charged residues on the cytoplasmic side. Into the ROCKET scaffold, we designed several CDL binding sites based on our observations from *E. coli* proteins and tested their effect on tetramer stability using nMS. We find that local dynamics and the spatial distribution of charged residues distinguish stabilizing from non-stabilizing sites. However, we also observe that predicting the impact of individual mutations on lipid binding and stabilization from the structure can be challenging, even in our highly artificial system. These difficulties arise from the fact that lipid interactions are heterogeneous, and the loss of one type of contact may be compensated by another. Screening our database of *E. coli* CDL binding sites (<https://osf.io/gftqa/> [↗](#)) for binding sites that resemble stabilizing sites in ROCKET, we uncover a highly stabilizing CDL interaction in the membrane protease GlpG, which regulates the substrate preference of the enzyme. In summary, our study demonstrates the potential as well as the challenges in designing functional CDL sites on artificial proteins that can recognize membrane compositions.

Results

Design of a CDL binding site in ROCKET

As first step, we characterized the inherent lipid binding properties of ROCKET (Fig 1a [↗](#)) through CG-MD simulations of the protein in a mimetic *E. coli* membrane. The membrane composition was modeled with a distribution of POPE, POPG, and CDL in a ratio of 67:23:10, and the system was simulated for 5 x 10 μ s while monitoring the lipid interactions. We observed abundant lipid interactions, with CDL displaying markedly more localized binding than POPE or POPG (Fig S1). The N-terminal region on the first transmembrane helix bound CDL with average occupancy of 71 % and average residence time of 35 ns (at R9). These values are extracted from the full 50 μ s of simulation data. The site, which we termed Site 1, consists of three basic residues (R9, K10, and R13) and an aromatic residue (W12), which corresponds to a consensus CDL binding motif (Fig 1b [↗](#)) (16 [↗](#)). We also observed a second site, involving W12 in a slightly rotated conformation, and R66 on helix 2 of the neighboring subunit. This site, termed Site 2, exhibited significantly lower occupancy of 56 % and an average residence time of 35 ns (at R66). W12 can engage in CDL binding at either site, including simultaneously both sites (Movie S1). Both sites represent distinct lipid binding modes: Site 1 is a high-occupancy site away from the protein core with extensive head-group interactions, and Site 2 is a lower-occupancy site with extensive acyl chain contacts close to the protein core. Note that, while the occupancies are high, the residence times are relatively low, as CDL is readily exchanged between the two sites. We decided to use these two sites, which arose from purely statistical distribution of charged and aromatic residues, as basis for engineering a stabilizing CDL site.

To separate the two CDL binding modes, we generated ROCKET mutants *in silico* and performed CG-MD with two subunits each of ROCKET and ROCKET mutants. We found that substituting the charged residues of Site 1 with alanine (R9A/K10A/R13A, Fig 1c [↗](#)) redirected preferential CDL

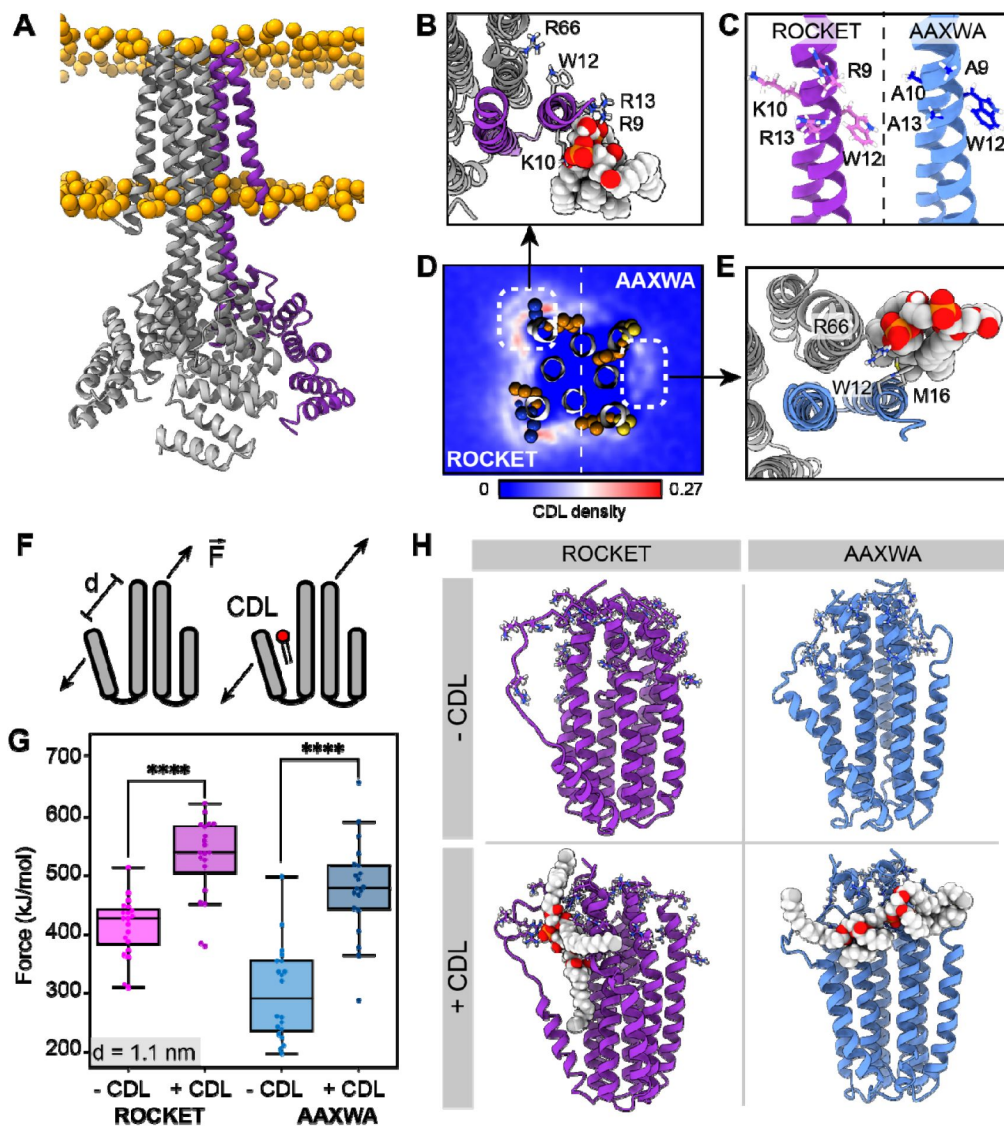


Fig 1.

ROCKET contains two CDL binding sites with different structural implications.

(A) Structure of ROCKET (PDB ID 6B85) in the membrane, with one protein subunit highlighted in purple and phosphate headgroups for the membrane shown as orange spheres. The structure was obtained from MemProtMD. (B) Top view of CDL binding to Site 1 taken from a 10 μ s snapshot of a CG-MD simulation. The poses were converted to atomistic using CG2AT2 (23) to the CHARMM36m force field (24). CDL is shown as spacefill, and residues R9, K10, W12, R13, and R66 as sticks. The CDL-binding subunit is highlighted in purple. (C) Design of the ROCKET^{AAXWA} variant. Site 1 on helix 1 of ROCKET is shown on the left (purple), and ROCKET^{AAXWA} with the mutations R9A/K10A/R13A on the right (blue). (D) CG-MD-derived CDL densities around a heterotetramer composed of two ROCKET subunits (left) and two ROCKET^{AAXWA} subunits (right). Units are number density. Site 1 on ROCKET and Site 2 on ROCKET^{AAXWA} are highlighted by dashed boxes. R9, K10, W12, and R13 are shown as spheres (basic in blue, aromatic in orange). Densities are computed over 5 $\times$ 10 μ s simulations. (E) Top view of CDL binding to Site 2 in ROCKET^{AAXWA} following CG-MD and converted to atomistic as per panel B. Interacting residues W12, M16, and R66 on the neighboring subunit (grey) are shown as sticks. (F) Setup of gas-phase MD simulations for unfolding of ROCKET and ROCKET^{AAXWA} with and without lipids. The placement of the lipid in the schematic is arbitrary. (G) Plots of the integral of the force required to separate helix 1 and 2 ($d = 1.1$ nm), for ROCKET (purple) ($p = 3.85 \times 10^{-7}$) and ROCKET^{AAXWA} (blue) ($p = 2.93 \times 10^{-8}$) with and without bound CDL show a more pronounced increase in stability for CDL-bound ROCKET^{AAXWA} compared to ROCKET (two-tailed t-test with $n = 20$). (H) Snapshots from gas-phase MD simulations show broad interactions of CDL across the subunits of lipid-bound ROCKET^{AAXWA} (blue) and more localized interactions with fewer intermolecular contacts for ROCKET (purple). Amino acid position 9, 10, 12, 13, and 66 are shown as sticks in each subunit.

binding to Site 2 (**Fig 1d, e**). In this mutant, which we termed ROCKET^{AAXWA}, the Site 2 had an occupancy of 53 % and an increased average residence time of 47 ns (R66), whereas Site 1 had a reduced occupancy of 45 % and a residence time of 45 ns (R9A). This occupancy difference was quantified by CG simulations and showed significant reduction of total CDL binding between ROCKET and ROCKET^{AAXWA} (**Fig 1d**, S1).

Next, we evaluated the potential for lipid-mediated stabilization at both sites using gas-phase atomistic MD simulations, which allows for a direct comparison with nMS. We applied a pulling force between two adjacent subunits of ROCKET and ROCKET^{AAXWA} tetramers with and without bound CDL and determined the force required to separate the protein chains (**Fig 1f**). Analysis of the secondary structure content shows that the AAXWA mutation stabilizes the conformation of helix 1 (**Fig S2**). However, we found that separating the adjacent helices from the neighboring subunits of ROCKET^{AAXWA} by 1.1 nm, the point at which non-covalent interactions between the transmembrane helices are disrupted, required more force when CDL was present (**Fig 1g**, **Fig S2**). ROCKET, on the other hand, displayed a lower significant difference in force with or without CDL. Snapshots from the simulations reveal that the lipid forms multiple contacts with both subunits adjacent to Site 2 in ROCKET^{AAXWA}, which likely gives rise to the stabilizing effect (**Fig 1h**). We conclude that channeling the CDL molecules to inter-helix sites may be a prerequisite for lipid-mediated stabilization.

Inter-helix CDL binding stabilizes ROCKET^{AAXWA} in the gas-phase

Having derived two ROCKET variants with distinct CDL binding modes from MD simulations, we turned to cryogenic electron microscopy (cryo-EM) and nMS to investigate their lipid interactions experimentally. We first analyzed ROCKET and ROCKET^{AAXWA} in the presence of CDL by cryo-EM. The resulting density maps for each protein with no other particle class detected, refined to a resolution of 3.8 and 3.9 Å respectively, show essentially identical architectures that agree with the previously solved crystal structure of ROCKET (22), confirming that the mutations do not disrupt the native structure (**Fig S3**, Table S1). Although a definitive atomic-level molecular model was not possible at this resolution, we also observed in both maps a diffuse non-protein density which partially overlaps the head-group of CDL predicted in Site 2 (**Fig S3**). Interestingly, we see no extra density in Site 1, however, this site is more exposed, making it more likely that excess detergent can outcompete the binding of CDL in this site. Furthermore, the orientation of CDL is more flexible in Site 1 than Site 2 (**Figure 1D**), which also reduces the likelihood of obtaining a sufficiently defined density. To determine lipid binding preferences, we therefore reconstituted the proteins into liposomes composed of polar *E.coli* polar lipid extracts (**Fig 2a**). By releasing the proteins from the liposomes inside the mass spectrometer and monitoring the intensity peaks corresponding to apo- and lipid-bound protein, we can compare lipid preferences of both variants (**Fig 2a**). We find that tetrameric ROCKET retains up to three CDL molecules, which can be identified by their characteristic 1.4 kDa mass shift, as well as a significant number of phospholipids between 700 and 800 Da (**Fig 2b**). The data thus show a preference for CDL, which constitutes only 10% of the liposome. Interestingly, nMS of ROCKET^{AAXWA} revealed a similarly specific retention of up to three CDL molecules for 17+ charge state, although the intensity of the lipid adducts was reduced by approximately 50% (**Fig 2c**). The mass spectra show that the preference for CDL is preserved in the ROCKET^{AAXWA} variant, while the occupancy is reduced, indicating either lower affinity in solution or lower stability of the protein-lipid complex in the gasphase.

To validate these findings, we analyzed lipid binding to detergent-solubilized ROCKET. First, we optimized detergent conditions for maintaining the intact ROCKET tetramer (**Fig S4**). Importantly, we observe no co-purified lipids, indicating that detergent can remove CDL during extraction, as expected for lipids with short residence times.(21) We then performed a competition assay where we mixed both variants in C8E4 detergent containing a limiting amount of CDL (50 μM) and monitored binding with nMS. As expected, both variants bound three distinguishable CDL

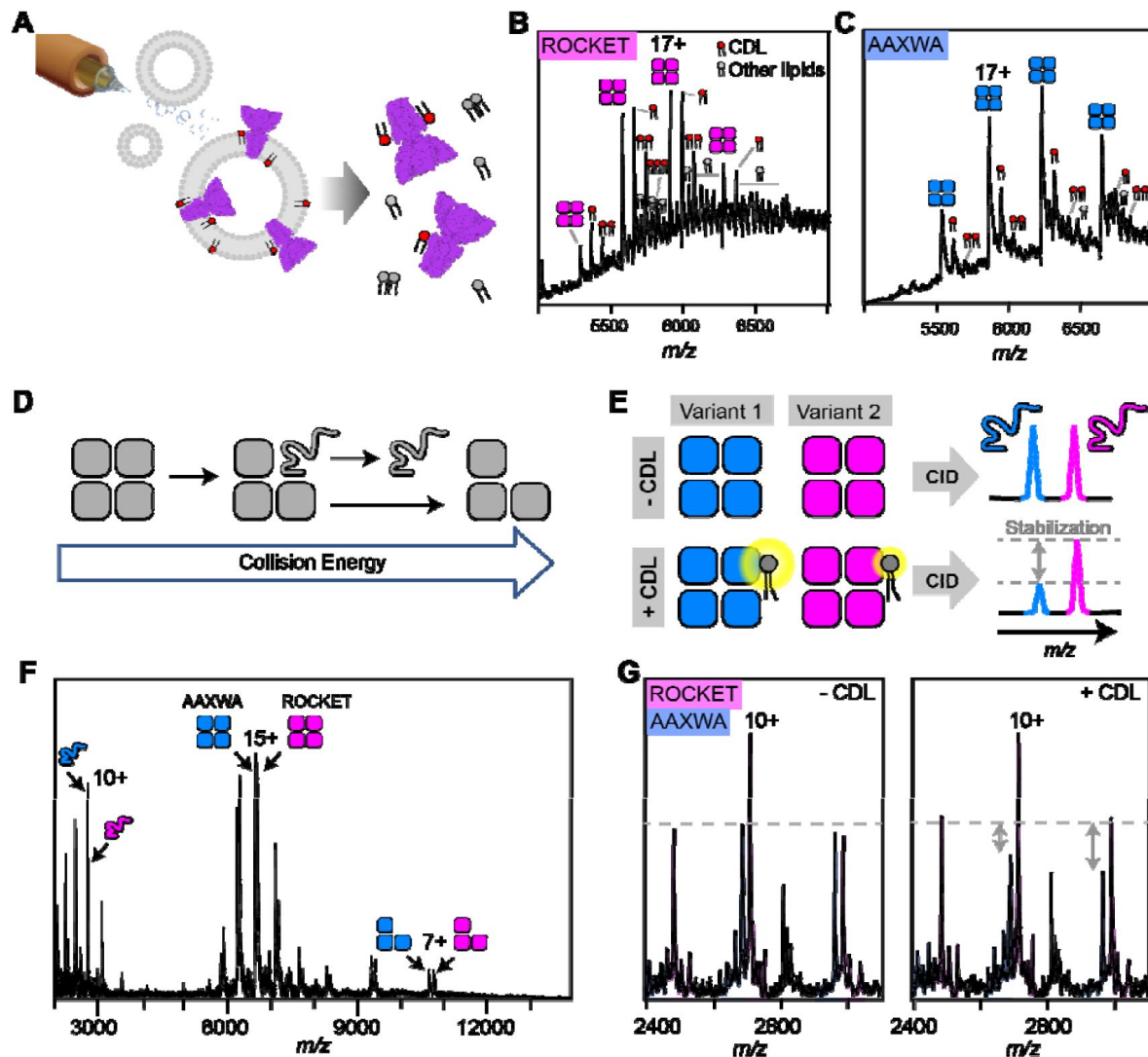


Fig 2.

nMS analysis of lipid binding and lipid mediated stabilization of ROCKET and ROCKET^{AAXWA}.

(A) Schematic depiction of electrospray ionization (ESI) process for proteo-liposomes, leading to the ejection of protein-lipid complexes into the gas-phase. (B) A representative mass spectrum of ROCKET released from proteoliposomes shows tetramers with 1-3 bound CDL molecules, as judged by the characteristic mass shift of 1.4 kDa, as well as additional lipids with molecular weights between 700 and 800 Da. (C) Release of ROCKET^{AAXWA} from proteoliposomes shows retention of CDL molecules. For the 18+ ion of the tetramer, a maximum of three CDL adducts can be assigned unambiguously. The reduced lipid adduct intensity compared to ROCKET indicates reduced lipid binding and/or complex stability. (D) Schematic illustrating the process of gas-phase subunit unfolding and ejection from ROCKET tetramers at increasing collision energies. (E) A nMS assay to assess CDL-mediated stabilization of ROCKET and ROCKET^{AAXWA}. Simultaneous dissociation of ROCKET and ROCKET^{AAXWA} leads to the ejection of unfolded monomers, which can be quantified by nMS (top row). Addition of CDL to the same mixture results in lipid binding to tetramers. If CDL binding stabilizes one tetrameric variant more than the other, the amount of ejected monomers will be reduced accordingly (bottom row). (F) Representative mass spectrum of ROCKET and ROCKET^{AAXWA} at a collision voltage of 220 V. Intact tetramers are seen in the middle, ejected monomers and stripped trimers are seen in the low and high m/z regions, respectively. (G) Zoom of the low m/z region of a mixture of 25 μ M each of ROCKET and ROCKET^{AAXWA} with a collision voltage of 200 V before (left) and after (right) the addition of 50 μ M CDL. The three main charge states for both variants can be distinguished based on their mass difference. Addition of CDL reduces the intensity of the ROCKET^{AAXWA} monomer peaks compared to ROCKET (dashed line).

molecules per tetramer, however, ROCKET displayed significantly more intense lipid adducts than ROCKET^{AAXWA} (Fig S4). In CG-MD simulations, the overall CDL occupancy is lower in ROCKET^{AAXWA} than in ROCKET, meaning fewer lipids will be bound simultaneously. The nMS data show CDL retention by both variants, but the ROCKET^{AAXWA} protein has lower-intensity CDL adduct peaks (**Figure 2B, C**). This finding suggests that both variants bind CDL, but in the ROCKET^{AAXWA} variant, the sites have lower occupancy. The nMS data are therefore consistent with CDL binding preferentially to Site 1 in ROCKET and preferentially to Site 2 in the ROCKET^{AAXWA} variant.

Next, we explored how CDL binding to either site affects the stability of ROCKET, using the oligomeric state in nMS as a measure. To avoid interference from different lipids in the reconstituted liposome system, we switched to detergent micelles as vehicles for nMS and employed gas-phase dissociation of the intact protein complexes to remove bound detergent. Briefly, collisions with gas molecules in the ion trap of the mass spectrometer cause thermal unfolding of a single subunit in the complex, which is then ejected as a highly charged, unfolded monomer (**Fig 2d**) (25). By comparing the peak intensities of the monomers that are ejected simultaneously from two protein oligomers, we can obtain information about their relative stabilities. Therefore, by adding CDL to an equimolar mixture of ROCKET and ROCKET^{AAXWA}, dissociating the resulting complexes, and monitoring changes in monomer signal intensities, we can determine whether lipid binding to Site 1 or Site 2 affects tetramer stability (**Fig 2e**). Importantly, by comparing changes in peak intensities with and without CDL while keeping all other conditions constant, we can avoid interference from changes in gas-phase fragmentation or ionization efficiency. nMS of ROCKET and ROCKET^{AAXWA} shows the release of highly charged monomers which can be distinguished based on their masses (**Fig 2f**). We then added CDL to the protein solution and repeated the measurement using identical conditions. We observed a reduction in the peak intensities of ROCKET^{AAXWA} monomers compared to ROCKET (**Fig 2g**). We do not observe a change in the charge state distributions for tetramers or monomers, or notable fragmentation. Therefore, the change in monomer ratio suggests that CDL stabilizes the ROCKET^{AAXWA} tetramer to a greater extent than the ROCKET tetramer. These findings are surprising, since the AAXWA variant displays significantly lower lipid binding (**Fig 2 B, C**). However, considering the predictions from CG- and gas-phase MD, the increase in stability can be attributed to the preferential binding of CDL to the inter-helix Site 2 in the AAXWA variant. CDL binding to the distal Site 1, as preferred in ROCKET, involves fewer intermolecular contacts, and is therefore unlikely to exhibit a similarly stabilizing effect.

Multiple structural features impact CDL-mediated stabilization

The finding that inter-helix CDL binding stabilizes a tetrameric membrane protein in the gas-phase recapitulates a key feature of both prokaryotic and eukaryotic membrane proteins (18, 26). Unlike naturally evolved proteins, however, the extraordinary stability and mutation tolerance of the ROCKET scaffold enables us to dissect further the requirements for CDL-mediated stabilization. We therefore applied the above MS strategy to quantitatively assess lipid-mediated stabilization in our model system between two protein variants using ROCKET^{AAXWA} as an internal reference. We can determine relative stability changes upon CDL addition for different ROCKET variants by plotting the ratios of the total intensity of the peaks for monomeric ROCKET mutants (ROCKET^{MUT}) to the total intensity of all protein monomer peaks in the spectrum (ROCKET^{MUT} + ROCKET^{AAXWA}) with and without CDL. If ROCKET^{AAXWA} is stabilized more than the variant of interest, the ratio increases with CDL addition (**Fig 3a**). As expected, the AAXWA mutation significantly increased the stabilizing effect of CDL, as determined from four independent repeats (**Fig 2g, 3b, and 3c**). With this assay, we then explored whether introducing different structural features into Site 1 could turn it into a stabilizing CDL binding comparable to Site 2. As a first hypothesis, we reasoned that a destabilization of the core of ROCKET might increase the effect of CDL. We introduced a destabilizing mutation (A61P) in helix 2, right below the headgroup region, theorizing that the proline-induced kink would destabilize the ROCKET tetramer. AlphaFold2 predictions (27, 28) indicated that the ROCKET^{A61P} mutation

does not affect the tetrameric state (29 [↗](#)), which was confirmed by nMS. To our surprise, ROCKET^{A61P} exhibited significantly less CDL stabilization than ROCKET^{AAXWA}, and was comparable to ROCKET (Fig 3d [↗](#), Fig S5). This observation indicates that the introduction of a proline in the protein core does not sufficiently destabilize the protein to be counteracted by lipid binding.

As second hypothesis, we reasoned that additional lipid binding sites may increase the effect of CDL binding (Fig S6). We therefore mutated residue D7 to alanine and S8 to arginine. The D7A/S8R variant retains the high-affinity Site 1 on helix 1, but includes an additional site composed of R8 on helix 1 and R66 and E68 on helix 2 which does not overlap with Site 1 (Fig 3d [↗](#)). Quantification of the monomer release with and without CDL suggests a shift towards increased stability with CDL, albeit not as pronounced as for ROCKET^{AAXWA} (Fig 3d [↗](#), Fig S5). This data leads us to speculate that Site 1 may still bind the bulk of the available CDL molecules, or that the salt bridge S8-E68 may contribute increased stability in a CDL-independent manner.

Having explored directed lipid binding (AAXWA), increased lipid binding (D7A/S8R) and core destabilization (A61P), we reasoned that the flexibility of the CDL binding site may affect stabilization. This feature is challenging to implement in the ROCKET scaffold, since it is designed around a tightly folded hydrogen bond network with a melting temperature of > 90°C (22 [↗](#)). We therefore decided to untether helix 1 from the core of the protein by mutating R66, which forms a salt bridge with D7, to alanine. The AlphaFold2 model shows helix 1 being tilted away from the core, creating a large hydrophobic gap in the transmembrane region and turning Site 1 towards the neighboring subunit (Fig 3f [↗](#), Fig S5). Interestingly, ROCKET^{R66A} showed lower signal intensities in native mass spectra than all other variants, which may indicate overall lower stability in detergent. Quantification of the monomer release with and without CDL revealed no significant difference compared to ROCKET^{AAXWA}, which means CDL binding has a stabilizing effect on both proteins (Fig 3f [↗](#)). This finding is surprising, since loss of R66 should result in increased binding to Site 1 and thus not stabilize the protein. However, untethering helix 1 may create an opportunity for CDL coordinated by W12 and/or R9-R13 to insert its acyl chains into the resulting inter-helix gap. In this manner, CDL could exert a stabilizing effect in the absence of preferential headgroup interactions.

From the designed ROCKET variants, we can conclude that structure-based predictions of stabilizing CDL interactions is challenging, as they arise from a combination of headgroup- and acyl chain interactions, as well as from their impact on the local structural dynamics of the protein. However, from our observations, we can conclude that CDL binding involving different helices, as in ROCKET^{AAXWA}, and connecting flexible regions, as in ROCKET^{R66A}, gives rise to the most pronounced CDL stabilization of our system (Fig 3g [↗](#)). Core destabilization, as well as introduction of additional headgroup contacts, had less of an impact, although the specific properties of the engineered protein scaffold may mitigate potential effects to some extent.

Identification of a stabilizing CDL binding site in the *E.coli* rhomboid intramembrane protease GlpG

As outlined above, the features that cause CDL-mediated stabilization of the ROCKET scaffold were designed based on observations from CG-MD investigation of CDL interactions with of *E. coli* membrane proteins. We therefore asked whether the same features could indicate stabilizing, and by extension, functionally relevant CDL interactions in naturally occurring proteins. To test this hypothesis, we evaluated our database of monomeric *E. coli* membrane proteins CDL sites (<https://osf.io/gftqa/> [↗](#)) for the sequence distribution of basic residues to find binding sites that span multiple helices. We reasoned that if two or more basic residues that interact with the same CDL molecule are located further apart in the sequence than approximately 30 positions, they have a high likelihood of being on separate helices, whereas sites spanning less than 10 residues are confined to a single helix or loop. Plotting the maximum sequence distance on a log scale reveals a

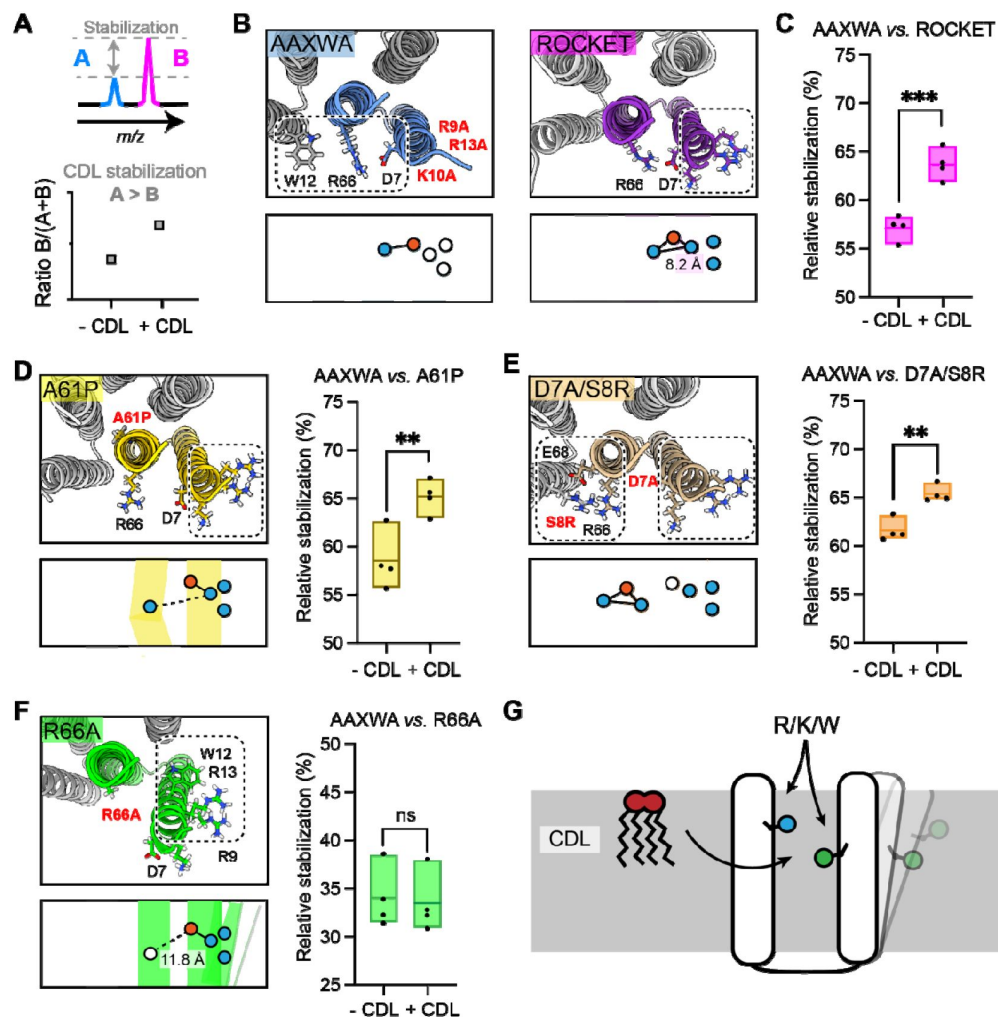


Fig 3.

Assessment of lipid-mediated stabilization effects on ROCKET variant.

(A) Principle for pairwise analysis of protein stabilization by CDL. Peaks representing monomers released of protein A and B display intensity changes upon lipid addition. Plotting the peak intensities as a ratio of B to total protein (A + B) shows an increase upon CDL addition, if A is stabilized more than B. (B) Residues involved in CDL headgroup binding to ROCKET and ROCKET^{AAXWA} were derived from CG-MD simulations (Fig 1) and are shown based on AlphaFold2 models as top view, with the area occupied by CDL as a dashed rectangle. A single subunit is colored (purple for ROCKET and blue for ROCKET^{AAXWA}). Below the structure, the orientation of the CDL site and the helices are shown as schematics. Positively and negatively charged residues are blue dots, respectively. Residues mutated to alanine are shown as white dots. (C) Plotting the peak intensity ratios of ROCKET to (ROCKET^{AAXWA} + ROCKET) in the presence and absence of CDL shows a decrease in ROCKET^{AAXWA} monomers when CDL is added ($p=0.0007$, two-tailed t-tests with $n=4$). (D) The CDL binding site and location of the A61P mutation (yellow) mapped on the AlphaFold2 model of ROCKET^{A61P} and shown as a schematic as a side view below. Intensity ratios show significantly more pronounced stabilization of ROCKET^{AAXWA} than ROCKET^{A61P} ($p=0.0084$, two-tailed t-tests with $n=4$). (E) Introduction of a second CDL binding site in the D7A/S8R variant (orange) mapped on the AlphaFold2 model of ROCKET^{D7A/S8R} and shown as a schematic as a side view below. The shift in intensity ratios show that ROCKET^{AAXWA} is still stabilized to a greater extent ($p=0.0019$, two-tailed t-tests with $n=4$), but with a smaller margin than ROCKET or ROCKET^{A61P}. (F) The R66A mutation, designed to disconnect helix 1 from the tetrameric protein core, results in an outward rotation of the CDL binding site, as shown in the AlphaFold2 model (green) and the side view schematic. Intensity ratios show no change upon CDL addition, suggesting that ROCKET^{R66A} is stabilized to a similar extent as ROCKET^{AAXWA} ($p=0.8113$, two-tailed t-tests with $n=4$). (G) Conceptual diagram depicting structural features that promote CDL-mediated stabilization. Distributing the residues that interact with the lipid headgroup, usually basic and aromatic residues, between two helices, as well as involvement of flexible protein segments, indicated by an outward movement of the right helix, also enhances stabilization by CDL.

bimodal distribution, with 75 CDL sites spanning less than 30 positions, and 180 sites spanning more than 30 positions (**Fig 4a** [Fig S5](#)). Site 1 in ROCKET is in the first group, with four positions between R9 and R13. This approach does not consider interfacial CDL molecules in homo-oligomers, which may bind *via* single residues on different subunits. We therefore limited the dataset to monomeric proteins with CDL sites spanning >30 residues which we manually inspected to find CDL sites linking potentially flexible regions.

The rhomboid intramembrane protease GlpG from *E.coli* contains a CDL site between R92 on helix 1 and K167 on helix 4, as well as one aromatic residue on each (W98 and Y160). Importantly, R92 borders on a disordered linker region that connects helix 1 to an N-terminal cytoplasmic domain (**Fig 4b** [Fig S6](#)). These features suggest that CDL binding may have structural implications. We therefore measured whether any lipids imparted thermal stabilization in a GFP-based thermal shift assay ([17](#) [Fig S7](#)). Measuring the fraction of GFP-tagged soluble protein after heating to 63 °C in the presence of different lipids showed that phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), as well as sphingomyelin (SM) (which is not found in *E. coli*) had no stabilizing effect, whereas phosphatidyl glycerol (PG) and CDL resulted in near-complete protection from heat-induced precipitation (**Fig 4c** [Fig S8](#)).

When performing CG-MD of GlpG in the model *E. coli* bilayer, we observed that CDL bound exclusively to the predicted site between helix 1 and 4, with an occupancy of 96.2% with an average residence time of 170 ns, but no preferential POPG binding was observed anywhere on the protein (**Fig 4d** [Fig S9](#)). On the cytoplasmic side, the non-conserved residues W136 and R137 can potentially interact with CDL, however no increased CDL density was observed ([Figure S7](#)). The data suggest that the stabilizing effect of POPG in the thermal shift assay is due to binding to this site in the absence of CDL. The near-100% occupancy by CDL in the mixed bilayer MD simulations suggests that CDL outcompetes POPG when both lipids are present. To test whether GlpG binds CDL in the membrane, we used nMS analysis. We have previously noted a 1.4 kDa adduct on purified GlpG ([30](#) [Fig S10](#)) and monomeric GlpG with sufficient activation to remove DDM micelle. Collisional activation revealed preferential loss of the adduct as a single species, confirming that it is a single lipid molecule, *i.e.* CDL (**Fig 4e** [Fig S11](#)). These findings are in good agreement with the presence of four lipid tails in total, which likely stem from a single CDL in this location in the crystal structure of GlpG ([Fig S7](#)), although these were tentatively modeled as a PA and a PE molecule next to each other in the structure ([31](#) [Fig S12](#)). The apparent high specificity for CDL prompted us to investigate the effect of CDL and PG on GlpG protease activity. For this purpose, we used a cleavage assay with fluorescently labeled substrates that represent either a soluble “extramembrane” substrate (KSp63) or transmembrane helix (KSp96) peptides containing the same GlpG cleavage site ([32](#) [Fig S13](#), [33](#) [Fig S14](#)). We found that PG and CDL both have a positive effect on the cleavage of the soluble substrate KSp63 compared to detergent-only, with both lipids increasing the cleavage rates by approximately 40% (**Fig 4f** [Fig S15](#)). PG mildly reduced the cleavage rate of the transmembrane substrate KSp96 to approximately 90% compared to detergent-only. Strikingly, CDL caused drastic inhibition of transmembrane substrate cleavage, with only 20% activity remaining (**Fig 4f** [Fig S15](#)). To find out how the lipid binding site, which is located on the cytoplasmic side, can affect substrate access to the active site, which faces the periplasm, we performed CG-MD simulations of the open and closed conformations of GlpG ([34](#) [Fig S16](#)). We found that both conformations bound CDL in the same place, which agrees with the observation that CDL affects the flexibility of the linker connecting the N-terminal domain ([34](#) [Fig S16](#)). Interestingly, the acyl chains of the tightly bound lipid extend into the gap between helices 2 and 5 ([Fig S7](#)), the lateral gate through which transmembrane substrates access the active site of GlpG ([34](#) [Fig S16](#)). Loop 5, whose movement is critical for catalytic activity, remains unaffected, providing a rationale for the selective inhibition of transmembrane substrate cleavage by CDL. Although the biological role of CDL-regulated substrate preferences of GlpG remains to be clarified, our results demonstrate the identification of a functionally relevant CDL binding site in an *E. coli* membrane protein based on the insights from artificial protein design.

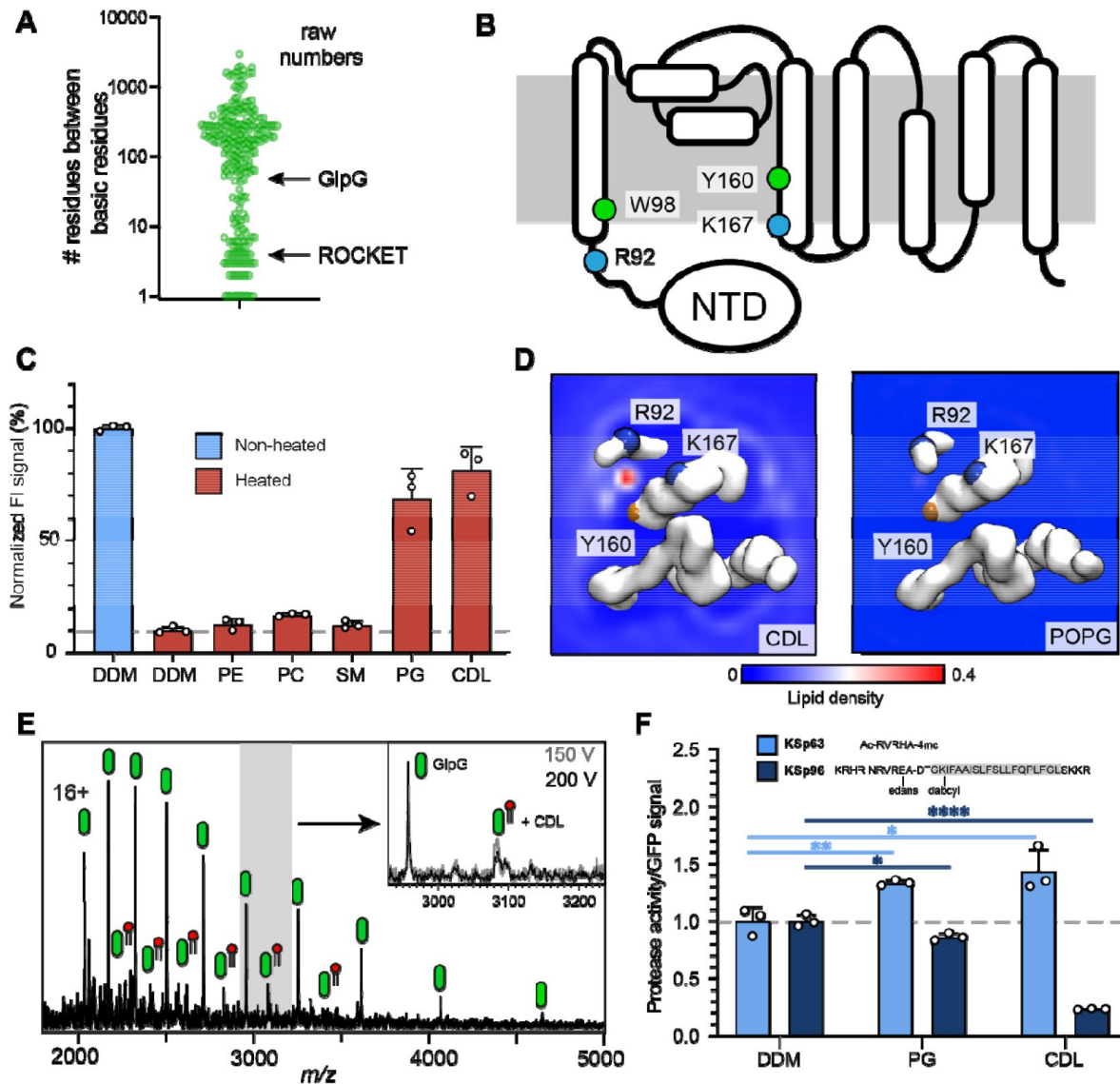


Fig 4.

Identification of a functional cdl binding site in the glpg membrane protease.

(a) distances between basic residues within the same cdl binding site and their occurrence in *e. coli* membrane proteins. GlpG contains a cdl binding site with two basic residues separated by 75 positions. Site 1 in rocket (four positions) is indicated for reference. (b) topology diagram of glpg indicating the locations of basic (r; arginine, l; lysine) and aromatic (w; tryptophan, y; tyrosine) residues that interact with cdl. (c) gfp-thermal shift assay of glpg in detergent or in the presence of pe, pc, sm, pg, or cdl. The average fluorescence intensity (fi) indicates the fraction of soluble protein before (unheated) or after heating to 63 °c and removing precipitated protein. Data are normalized against the non-heated control in detergent. All measurements were performed in triplicates (n = 3). (d) cg-md-derived lipid density plots for cdl (left) and popg (right) around the transmembrane region of glpg (pdb id 2ic8) viewed from the cytoplasmic side. Units are number density. Cdl, but not popg, exhibits preferential binding to the r92-k167 site. The backbones of r92, k67, and y160 are shown as spheres (basic in blue, aromatic in orange). (e) nms spectra of glpg released from detergent micelles show a 1.4 kda adduct which can be removed through collisional activation of the protein. (f) glpg proteolytic cleavage rates (specific activity, by normalizing to the gfp signal) for soluble (extramembrane) substrate (ksp63) or transmembrane substrate (ksp96, transmembrane helix shaded) in the presence of pg or cdl. Both lipids increase the cleavage rate for the soluble substrate by approx. 40%. For the transmembrane substrate, addition of pg causes a moderate reduction in cleavage activity, whereas cdl causes near-complete inhibition of cleavage (cdl ksp96 $p < 0.0001$, cdl ksp63 $p = 0.028$, pg ksp96 $p = 0.0171$, and pg ksp63 $p = 0.0089$, all analyzed with two-tailed t-test, n=3).

Discussion

In this study, we have systematically investigated the requirements for CDL binding and stabilization of membrane proteins, using the scaffold protein ROCKET as a model system. CDL binding sites have few sequence requirements, which is evident from the fact that ROCKET contains multiple interaction sites solely from statistical distribution of aromatic and charged amino acids at the membrane interface (**Fig 1**). These sites exhibit characteristic features, including electrostatic interactions with the negatively charged lipid headgroups and flanking aromatic residues that align the acyl chains with the hydrophobic transmembrane domain. By introducing mutations at these sites, we can probe their contribution to CDL-mediated stabilization with native MS. To our surprise, we find that although CDL binding sites share well-defined structural features, mutations of these features does not always yield predictable structural effects. Instead, their ability to contribute to lipid-mediated stabilization depends on multiple additional factors. For example, mutations that reduce CDL binding to a high-affinity site can cause redistribution to lower-affinity sites that have, in turn, a more pronounced effect on stability. This appears to be the case for *ROCKET*^{AAXWA}. Furthermore, mutations that reduce lipid binding at a low-affinity site may change the local stability of the protein, and results in an increase in lipid-mediated stabilization, as seen in *ROCKET*^{R66A}. Lipid interactions are dynamic and heterogeneous, and mutating a well-defined CDL site can give rise to multiple compensatory interactions which in turn affect the local protein environment.

Despite these challenges, the different CDL binding sites in ROCKET demonstrate that commonly observed structural features (the number of headgroup-interaction residues, local flexibility, and the involvement of multiple transmembrane helices) impact lipid-mediated stabilization to different extent. Building upon the insights garnered from ROCKET, we extended our investigation to *E. coli* proteins and identified GlpG as a case study for CDL's impact on function. Previous studies have shown that GlpG conformation is impacted by the surrounding lipids and membrane geometry (30, 31), and CDL has been suggested to affect proteolytic activity (35). Our observation that CDL acts as an allosteric activator for the cleavage of soluble substrates while exerting an inhibitory effect on the processing of transmembrane substrates establishes CDL as a regulator of GlpG activity. Similarly, a CDL binding site (W70/R71/R151) in the yeast ATP/ADP carrier Aac2 has the same hallmarks as the GlpG site (Figure S8). CDL binding to this site connects the flexible N-terminal transmembrane helix to the protein core, resulting in stabilization of the tertiary structure and increased transport activity. nMS shows that the protein retains co-purified CDL. (14) These findings suggest that the features that mediate lipid stabilization in ROCKET are hallmarks of functionally important lipid binding sites in native membrane proteins.

Although we can identify relatively intuitive features of CDL interaction sites, we find that the connection between lipid binding and stabilization is not clear-cut. For example, one destabilizing mutation increases lipid-mediated stabilization whereas another does not (compare *ROCKET*^{R66A} and *ROCKET*^{A61P}). Furthermore, being able to bind more lipids do not translate to forming more stable complexes (compare ROCKET and *ROCKET*^{AAXWA}). The reasons are likely two-fold: Firstly, our approach has methodological limitations, as gas-phase stability is not easily correlated with condensed phase stability. In case of CDL, increasing the number of molecular contacts likely translates to stabilizing effects in both phases. However, charge interactions are relatively strengthened in the gas phase, whereas some hydrophobic contacts will be lost. For example, CDL sites contain aromatic residues (W or Y) close to the ester bonds of the lipids, which likely serve to orient the lipids, but the roles of which have not been examined in ROCKET. Unlike charge interactions with lipid head groups, such subtle contributions are likely distorted by the transfer to the gas phase, making it difficult to confidently assign changes in stability or lipid occupancy. Furthermore, the bulk of the nMS analysis is done using detergents, which have delipidating

effects, meaning we will observe fewer lipid binding events and lower occupancy in nMS than in the CG-MD simulations.⁽²¹⁾ Collisional activation required to remove the detergent micelle or lipid vesicle additionally strips away lipids that bind predominantly via hydrophobic interactions.

To avoid this limitation of our MS approach, we have focused here CDL bound via direct headgroup contacts, which can be readily predicted with CG-MD and analyzed with nMS. Secondly, our approach highlights that protein-lipid interactions are complex. The ROCKET scaffold is, *per design*, extremely stable and has no function. Thus, it does not capture the dynamic nature of most membrane proteins, which, in turn, is often related to lipid-mediated regulation⁽¹²⁾. The key finding of our study is that emulating the architecture of a binding site is only the first step to uncovering the principles of lipid regulation, and that the effect of lipids on the local dynamics are critical to understanding how they shape membrane protein function. Integrated design approaches based not only on static structures but on dynamic models are therefore the key to designing membrane proteins with membrane-specific functions.

In summary, we present a protein design-driven approach to decipher mechanisms by which CDL regulates membrane protein stability. Our findings illuminate critical protein features that are challenging to predict or design *de novo*. Further integration of MD simulations and nMS with protein engineering could help to overcome some of the challenges identified here, such as the impact of local protein flexibility, and offer a promising pathway to uncover novel CDL binding sites. The findings not only contribute to our understanding of lipid-protein interactions but highlight potential avenues for the design of membrane proteins with tailored stability and function, potentially informing therapeutic strategies targeting membrane protein dysfunctions.

Data and materials availability

Cryo-EM density maps of ROCKET and ROCKET^{AAXWA} in detergent micelles have been deposited in the Electron Microscopy Data Bank under accession number EMD-50106 and EMD-50107 respectively. All data are available in the main text or the supplementary materials.

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Additional files

Supporting information [↗](#)

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Reviewer #1 (Public review):

Summary:

The study combines predictions from MD simulations with sophisticated experimental approaches including native mass spectrometry (nMS), cryo-EM, and thermal protein stability assays to investigate the molecular determinants of cardiolipin (CDL) binding and binding-induced protein stability/function of an engineered model protein (ROCKET), as well as of the native *E. coli* intramembrane rhomboid protease, GlpG.

Strengths:

State-of-the-art approaches and sharply focused experimental investigation lend credence to the conclusions drawn. Stable CDL binding is accommodated by a largely degenerate protein fold that combines interactions from distant basic residues with greater intercalation of the lipid within the protein structure. Surprisingly, there appears to be no direct correlation between binding affinity/occupancy and protein stability.

Overall, using both model and native protein systems, this study convincingly underscores the molecular and structural requirements for CDL binding and binding-induced membrane protein stability. This work provides much-needed insight into the poorly understood nature of protein-CDL interactions.

<https://doi.org/10.7554/eLife.104237.2.sa2>

Reviewer #3 (Public review):

Summary:

The relationships of proteins and lipids: it's complicated. This paper illustrates how cardiolipins can stabilize membrane protein subunits - and not surprisingly, positively charged residues play an important role here. But more and stronger binding of such structural lipids does not necessarily translate to stabilization of oligomeric states, since many proteins have alternative binding sites for lipids which may be intra- rather than intermolecular. Mutations which abolish primary binding sites can cause redistribution to (weaker) secondary sites which nevertheless stabilize interactions between subunits. This may be at first sight counterintuitive but actually matches expectations from structural data

and MD modelling. An analogous cardiolipin binding site between subunits is found in E.coli tetrameric GlpG, with cardiolipin (thermally) stabilizing the protein against aggregation.

Strengths:

The use of the artificial scaffold allows testing of hypothesis about the different roles of cardiolipin binding. It reveals effects which are at first sight counterintuitive and are explained by the existence of a weaker, secondary binding site which unlike the primary one allows easy lipid-mediated interaction between two subunits of the protein. Introducing different mutations either changes the balance between primary and secondary binding sites or introduced a kink in a helix - thus affecting subunit interactions which are experimentally verified by native mass spectrometry.

[Editors' note: The reviewers agreed that the authors addressed all reviewer comments adequately and rigorously.]

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Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public review):

Summary:

The study combines predictions from MD simulations with sophisticated experimental approaches including native mass spectrometry (nMS), cryo-EM, and thermal protein stability assays to investigate the molecular determinants of cardiolipin (CDL) binding and binding-induced protein stability/function of an engineered model protein (ROCKET), as well as of the native E. coli intramembrane rhomboid protease, GlpG.

Strengths:

State-of-the-art approaches and sharply focused experimental investigation lend credence to the conclusions drawn. Stable CDL binding is accommodated by a largely degenerate protein fold that combines interactions from distant basic residues with greater intercalation of the lipid within the protein structure. Surprisingly, there appears to be no direct correlation between binding affinity/occupancy and protein stability.

Weaknesses:

(i) While aromatic residues (in particular Trp) appear to be clearly involved in the CDL interaction, there is no investigation of their roles and contributions relative to the positively charged residues (R and K) investigated here. How do aromatics contribute to CDL binding and protein stability, and are they differential in nature (W vs Y vs F)?

Based on the simulations in Corey et al (Sci Adv 2021), aromatic residues, especially tryptophan, appear to help provide a binding platform for the glycerol moiety of CDL which is quite flat. This interaction is likely why we generally see the tryptophan slightly further into the plane of the membrane than the basic residues, where it may help to orient the lipid. Unlike charge interactions with lipid head groups, such subtle contributions are likely distorted by the transfer to the gas phase, making it difficult to confidently assign changes in stability or lipid occupancy to interactions with tryptophan. We have added an explanation of these considerations to the Discussion section (page 13, last paragraph).

(ii) In the case of GlpG, a WR pair (W136-R137) present at the lipid-water on the periplasmic face (adjacent to helices 2/3) may function akin to the W12-R13 of ROCKET in specifically binding CDL. Investigation of this site might prove to be interesting if it indeed does.

Thank you for the suggestion. In our CG simulations, we don't see significant CDL binding at this site, likely because there is just a single basic residue. We note that there is a periplasmic site nearby with two basic residues (K132+K191+W125) with a higher occupancy, however still far lower than the identified cytoplasmic site. In general, periplasmic sites are less common and/or have lower affinity which may be related to leaflet asymmetry (Corey et al, Sci Adv 2021). We added the CDL density plot for the periplasmic side to Figure S7 and noted this on page 9, next-to-last paragraph.

(iii) Examples of other native proteins that utilize combinatorial aromatic and electrostatic interactions to bind CDL would provide a broader perspective of the general applicability of these findings to the reader (for e.g. the adenine nucleotide translocase (ANT/AAC) of the mitochondria as well as the mechanoenzymatic GTPase Drp1 appear to bind CDL using the common "WRG" motif.)

Several confirmed examples are presented in Corey et al (Sci Adv 2021), the dataset which we used to identify the CDL site in GlpG. So essentially, our broader perspective is that we test the common features observed in native proteins in an artificial system. While it is not clear how a peripheral membrane protein like Drp1 fits into this framework, the CDL binding sites in ANTs indeed have the same hallmarks as the one in GlpG (Hedger et al, Biochemistry 2016). We recently contributed to a study demonstrating that the tertiary structure of ANT Aac2 is stabilized by co-purified CDL molecules, underscoring the general validity of our findings (Senoo et al, EMBO J 2024). We have added this information to the discussion, pg 12, third paragraph, and added a figure (S8, see below) to highlight the architecture of the Aac2-CDL complex.

Overall, using both model and native protein systems, this study convincingly underscores the molecular and structural requirements for CDL binding and binding-induced membrane protein stability. This work provides much-needed insight into the poorly understood nature of protein-CDL interactions.

We thank the reviewer for the positive assessment!

Reviewer #2 (Public review):

Summary:

The work in this paper discusses the use of CG-MD simulations and nMS to describe cardiolipin binding sites in a synthetically designed, that can be extrapolated to a naturally occurring membrane protein. While the authors acknowledge their work illuminates the challenges in engineering lipid binding they are able to describe some features that highlight residues within GlpG that may be involved in lipid regulation of protease activity, although further study of this site is required to confirm it's role in protein activity.

Comments

Discrepancy between total CDL binding in CG simulations (Fig 1d) and nMS (Fig 2b,c) should be further discussed. Limitations in nMS methodology selecting for tightest bound lipids?

We thank the reviewer for pointing out that this needs to be clarified. We analyze proteins in detergent, which is in itself delipidating, because detergent molecules compete with the lipids for binding to the protein, an effect that can be observed in MS (Bolla et al, Angew Chemie Int. Ed. 2020). Native MS of membrane proteins requires stripping of the surrounding lipid vesicle or detergent micelle in the vacuum region of the mass spectrometer, which is done through gentle thermal activation in the form of high-energy collisions with gas molecules. Detergent molecules and lipids not directly in contact with the protein generally dissociate easier than bound lipids (Laganowsky et al, Nature 2014), however, the even loosely bound lipids can readily dissociate with the detergent, artificially reducing occupancy. The nMS data is therefore likely biased towards lipids bound tightly (e.g. via electrostatic headgroup interactions), however, these are the lipids we are interested in, meaning that the use of MS is suitable here. We have noted this in the Discussion, last paragraph on page 12.

Mutation of helical residues to alanine not only results in loss of lipid binding residues but may also impact overall helix flexibility, is this observed by the authors in CG-MD simulations? Change in helix overall RMSD throughout simulation? The figures shown in Fig. 1H show what appear to be quite significant differences in APO protein arrangement between ROCKET and ROCKET AAXWA.

For most of the study, we use CG with fixed backbone bead properties as well as an elastic network to maintain tertiary structure. This means that a mutation to alanine will have essentially no impact on the stability of the helix or protein in general in the CG simulations in the bilayer. It should be noted that Figure 1H shows snapshots from atomistic gas phase simulations with pulling force applied (see schematic in Figure 1F, as well as Figure S1 for ends-point structures), where we naturally expect large structural changes due to unfolding. We have analyzed the helix content in the gas-phase simulations and see that helix 1 in ROCKET unwinds within 10 ns but stays helical ca. 10 ns longer when bound to CDL. The AAWXA mutation stabilizes the helical conformation independently of CDL binding, but CDL tethers the folded helix closer to the core (see Figure 1 G and H). We have added this information to the results section and the plot below to Figure S2.

CG-MD force experiments could be corroborated experimentally with magnetic tweezer unfolding assays as has been performed for the unfolding of artificial protein TMHC2. Alternatively this work could benefit to referencing Wang et al 2019 "On the Interpretation of Force-Induced Unfolding Studies of Membrane Proteins Using Fast Simulations" to support MD vs experimental values.

We apologize for the confusion here. The force experiments are gas-phase all-atom MD. The simulations show that the protein-lipid complex has a more stable tertiary structure in the gas phase. Since these are gas-phase simulations, they cannot be corroborated using in-solution measurements. Similarly, the paper by Wang et al is a great reference for solution simulations, however, to date the only validations for gas-phase unfolding come from native MS.

Did the authors investigate if ROCKET or ROCKETAAXWA copurifies with endogenous lipids? Membrane proteins with stabilising CDL often copurify in detergent and can be detected by MS without the addition of CDL to the detergent solution. Differences in retention of endogenous lipid may also indicate differences in stability between the proteins and is worth investigation.

We have investigated the co-purification of the ROCKET variants and did not observe any co-purified lipids (see Figure S4) which we clarified in the results section (page 5, third paragraph) now. We previously showed that long residence times in CG-MD are linked to the

observation of co-purified lipids, because they are not easily outcompeted by the detergent (Bolla et al, Angew Chemie Int. Ed. 2020). In CG-MD of ROCKET, we see that although the CDL sites are nearly constantly occupied, the CDL molecules are in rapid exchange with free CDL from the bulk membrane. For MS, all ROCKET proteins were extracted from the E. coli membrane fraction with DDM, which likely outcompetes CDL. This interpretation would explain why we see significant CDL retention when the protein is released from liposomes, but not when the protein is first extracted into detergent. For GlpG, CDL residence times in CG-MD are longer, which agrees with CDL co-purification. Similarly, there is clearly an enrichment of CDL when the protein is extracted into nanodiscs (Sawczyk et al, Nature Commun 2024).

Do the AAXWA and ROCKET have significantly similar intensities from nMS? The AAXWA appears to show slightly lower intensities than the ROCKET.

We did not observe a significant difference, however, in most spectra, the AAXWA peaks have a lower intensity than those of the other variants (see e.g. Figure S5). While this could be batch-to-batch variations, there may be a small contribution from the lower number of basic residues (see Abramsson et al, JACS au 2021). However, there is an excess of basic residues in the soluble domain of ROCKET, so this interpretation is speculative.

Can the authors extend their comments on why densities are observed only around site 2 in the cryo-em structures when site 1 is the apparent preferential site for ROCKET.

We base the lipid preference of Site 1 > Site 2 on the CG MD data, where we see a higher occupancy for site 1. At the same time, as noted in the text, CDL at both sites have rather short residence times. When the protein is solubilized in detergent, these times can change, and lipids in less accessible sites (such as cavities and subunit interfaces) may be subject to a slower exchange than those that are fully exposed to the micelle (Bolla et al, Angew Chemie Int. Ed. 2020). We speculate that this effect may favor retaining a lipid at site 2. Furthermore, site 1 is flexible, with CDL attaching in various angles while site 2 has more uniform CDL orientations (see CDL density plot in Figure 1D). EM is likely biased towards the less flexible site. Notably, the density is still poorly defined, so it is possible that a more variable lipid position in site 1 would not yield a notable density at all. We have added this information to the Results section (page 5, second paragraph).

The authors state that nMS is consistent with CDL binding preferentially to Site 1 in ROCKET and preferentially to Site 2 in the ROCKET AAXWA variant, yet it unclear from the text exactly how these experiments demonstrate this.

As outlined in the previous answer, we base our assessment of the sites on the CG MD simulations. There, we note that CDL binds predominantly to site 1 in ROCKET and predominantly to site 2 in AAXWA, however, the overall occupancy is lower in AAXWA than in Rocket, meaning fewer lipids will be bound simultaneously in that variant. The nMS data show CDL retention by both variants when released from liposomes, but the AAXWA has lower-intensity CDL adduct peaks (Figure 2B, C). We interpret this that both have CDL sites, but in the AAXWA variant, the sites have lower occupancy. We agree that this observation does not demonstrate that the CG MD data are correct, however, it is the outcome one expects based on the simulations, so we described it as “consistent with the simulations”. We have rephrased the section to make this clear.

As carried out for ROCKET AAXWA the total CDL binding to A61P and R66A would add to supporting information of characterisation of lipid stabilising mutations.

We considered this possibility too. Unfortunately, the mass differences between A61P / R66A and AAXWA are slightly too high to unambiguously resolve CDL adducts of each variant, as the 1st CDL peak of AAXWA partially overlaps with the apo peak of A61P or R66A.

Did the authors investigate a double mutation to Site 2 (e.g. R66A + M16A)?

While designing mutants, we tested several double mutants involving the basic residues that bind the CDL headgroups (e.g. R66 + AAXWA) but found that they could not be purified, probably because a minimum of positive residues at the N-terminus is required for proper membrane insertion and folding. M16 is an interesting suggestion, but wasn't considered because the more subtle effects of non-charged amino acids on CDL binding may be lost during desolvation (see also our response to Comment (i) from reviewer 1).

Was the stability of R66A ever compared to the WT or only to AAXWA?

Some of the ROCKET mutants have very similar masses that cannot be resolved well enough on the ToF instrument. While the R66-WT comparison is possible, we would not be able to compare it to R61P or D7A/S8R. To avoid three-point comparisons, we selected AAXWA as the common point of reference for all variants.

How many CDL sites in the database used are structurally verified?

At the time, 1KQF was the only verified *E. coli* protein with a CDL resolved in a high-resolution structure. The complex was predicted accurately, see Figure 6A in Corey et al (Sci Adv 2021), as were several non-*E. coli* complexes.

The work on GlpG could benefit from mutagenesis or discussion of mutagenesis to this site. The Y160F mutation has already been shown to have little impact on stability or activity (Baker and Urban Nat Chem Biol. 2012).

We thank the referee for their excellent suggestion. While Y160F did not have a pronounced effect, the other 3 positions of the predicted CDL binding site in GlpG have not been covered by Baker and Urban. Looking at sequence conservation in GlpG orthologs, manually sampling down to 50% identity (~1300 sequences in Uniprot) shows that Y160 and K167 are conserved, R92 varies between K/R/Q, whereas W98 is not conserved. The other (weak) site cited above (K132 and K191) is not conserved. A detailed investigation of how the conserved residues impact CDL binding and activity is already planned for a follow up study focusing on GlpG biology.

Reviewer #3 (Public review):

Summary:

*The relationships of proteins and lipids: it's complicated. This paper illustrates how cardiolipins can stabilize membrane protein subunits - and not surprisingly, positively charged residues play an important role here. But more and stronger binding of such structural lipids does not necessarily translate to stabilization of oligomeric states, since many proteins have alternative binding sites for lipids which may be intra- rather than intermolecular. Mutations which abolish primary binding sites can cause redistribution to (weaker) secondary sites which nevertheless stabilize interactions between subunits. This may be at first sight counterintuitive but actually matches expectations from structural data and MD modelling. An analogous cardiolipin binding site between subunits is found in *E. coli* tetrameric GlpG, with cardiolipin (thermally) stabilizing the protein against aggregation.*

“It’s complicated” We could not have phrased the main conclusions of our study better.

Strengths:

The use of the artificial scaffold allows testing of hypothesis about the different roles of cardiolipin binding. It reveals effects which are at first sight counterintuitive and are explained by the existence of a weaker, secondary binding site which unlike the primary one allows easy lipid-mediated interaction between two subunits of the protein. Introducing different mutations either changes the balance between primary and secondary binding sites or introduced a kink in a helix - thus affecting subunit interactions which are experimentally verified by native mass spectrometry.

Weaknesses:

The artificial scaffold is not necessarily reflecting the conformational dynamics and local flexibility of real, functional membrane proteins. The example of GlpG, while also showing interesting cardiolipin dependency, illustrates the case of a binding site across helices further but does not add much to the main story. It should be evident that structural lipids can be stabilizing in more than one way depending on how they bind, leading to different and possibly opposite functional outcomes.

We share the reviewer’s concern, as we clearly observe that TMHC4_R does not have the same type of flexibility as a natural protein. We find that by introducing flexibility, we start to see CDL-mediated effects. To test the validity of our findings from the artificial system, we apply them to GlpG. In response to a suggestion from Reviewer 1, we compared the findings to Aac2, and found that its stabilizing CDL site closely resembles that in GlpG (see new Figure S8).

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

Minor comments:

There are a number of typos/uncorrected statements in the text.

i) The last sentence of the Abstract appears to be an uncorrected mishmash of two.

ii) Line 66: "protects" should be just "protect"

iii) Line 75: Sentence appears to be incomplete. "...associated changes in protein stability." The word "stability" is missing.

We have made these changes.

iv) Fig. 2E. Are the magenta and blue colors inverted for variants 1 and 2?

No, the color is correct. greater stabilization of the blue tetramer (AAXAW) compared to WT (purple) will lead to fewer blue monomers than purple monomers in the mass spectrum.

v) Line 274: the salt bridge should be between R8-E68.

We have corrected this.

vi) Lines 350-354 (final sentence of the paragraph): The sentence does not read well (especially with the double negative element). Please reconstruct the sentence and/or

break it into two.

We have split the sentence in two.

Suggestions:

(i) While aromatic residues (in particular Trp) appear to be clearly involved in the CDL interaction, there is no investigation of their roles and contributions relative to the positively charged residues (R and K) investigated here. How do aromatics contribute to CDL binding and protein stability, and are they differential in nature (W vs Y vs F)?

See our response to comment (i) from reviewer 1. In short, subtle contribution to lipid interactions (such as pi stacking with Trp or Tyr) will likely be lost during transfer to the gas phase. However, see also our response to the last comment from reviewer 2, we plan to use solution-phase activity assays to investigate the effect of Trp on CDL binding to Glp. However, this is beyond the scope of the current study.

(ii) In the case of GlpG, a WR pair (W136-R137) present at the lipid-water on the periplasmic face (adjacent to helices 2/3) may function akin to the W12-R13 of ROCKET in specifically binding CDL. Investigation of this site might prove to be interesting if it indeed does.

We added the CDL density plot for the periplasmic side to Figure S7 and discuss further sites in GlpG in the Discussion section. See response to point (ii) above for details.

Reviewer #2 (Recommendations for the authors):

Minor comments

- Typo in abstract line 39-40
- Typo in figure legend of Fig 1 line 145
- Typo in line 149, missing R66 in residues shown as sticks description
- Lines 165-167 could benefit from describing what residues are represented as sticks

We have made these changes.

- Line 263 should refer to the figure where the tetrameric state was not affected by this mutation.

The full spectrum of the A61P mutant is not included in the figure, hence there is no reference,

- Addition of statistics to Fig. 4F ?

We have added significance indicators to the graph and information about the statistics to the legend.

Reviewer #3 (Recommendations for the authors):

Minor issues

l39: rewrite

We have made these changes.

160: provide evidence for what is presented as a general statement - cardiolipins might also regulate function without affecting oligomeric state, e.g. MtgA

This is a good point, we have added references to two examples where CDL work without affecting oligomerization (MtgA, Weikum et al BBA 2024, and Aac2, Senoo et al, EMBO J 2024).

174: not every functional interaction comes with a thermal shift

We use thermal shift as a proxy because it indicates tight interactions, even if they may not be functional. We have made this distinction clearer in the text.

178: this is true for electrostatic interactions such as are at play here, but not necessarily for hydrophobic ones

1133: in what direction is the pulling force applied - the figure seems to suggest diagonally?

The pull coordinate is defined as the distance between the centers of mass of the two helices. The direction of the pull coordinate in Cartesian coordinate space is thus not fixed.

fig 1f, 1159: "dissociating" meaning separation of subunits? the placement of the lipid within one subunit would not suggest that intermolecular interactions are properly represented here, please clarify

The lipid placement in the schematic is not representative since the lipid occupies different spaces in WT and AAXWA, we have noted this in the legend. Regarding line 159, "Dissociation" is not strictly correct, since the measure the force to separate helix 1 and 2, i.e. unfolding. We have changed the wording to "unfolding".

1173: was there any evidence in EM data for monomers or smaller oligomers?

No smaller particles were identified by visual inspection or in the particle classes. We have noted this in the methods section.

1203: were tetramer peaks isolated separately for CID?

C8E4 can cause some activation-dependent charge reduction, which could allow some tetramers to "sneak out" of the isolation window. We used global activation without precursor selection which subjects all ions to activation.

fig 2c: can you indicate the 3rd lipid binding as it seems to be in the noise

We can unambiguously assign the retention of three CDL molecules for 17+ charge state only, and clarified this in the legend to Figure 2.

fig3: can you pls clarify what is meant by stabilization here - less monomer in case A means a more stable oligomer, but "A > B" should lead to ratios < 50%. This does not help with understanding what "stabilization" means in panels c-f, please define what the y axis means for these. Please also explain the bottom panels (side view) in each case, what do the dots represent?

We apologize for the oversight of not explaining the side views, we have added a legend. The schematic in panel A is correct (compare the schematic in Figure 2 E). If tetramer A (blue) is

stabilized by CDL more than tetramer B “CDL stabilization A>B”), there will be fewer monomers ejected from A. If there is less A in the presence of CDL, then the ratio of B/(B+A) will go up.

It is not very clear what consequences the kink introduced by proline has for intra- vs. intermolecular interactions - the cartoons don't help much here

We agree, the A61P impact on the structure is subtle. The small kink it introduces is not really visible in the top view, and hence, we tried to emphasize this in the side view. We have clarified the meaning of the side view schematics in the legend.

I360: is that an assumption made here or is there evidence for displacement? native MS could potentially prove this.

This is an assumption based on the fact that we see very little binding of POPG in the mixed bilayer CG-MD. We have clarified this in the text. Measuring this with MS is an interesting idea, but we have no direct measurement of displacement, since addition of CDL and POPG to the protein in detergent would result in binding to other sites as well.

fig 4d: there is not much POPG density visible at all - why is that?

Both plots use the same absolute scale. There is simply much less POPG binding compared to CDL.

fig 4e: is this released protein already dissociated into monomers due to denaturation or excessive energy (CID product) - please comment.

The CID energy for the spectrum in Figure 4E was selected to show partial dissociation and monomer release at higher voltages (220V in this case). At lower voltages (150V-170V) we do not observe dissociation in C8E4, see Figure S4A.

I363: pls comment on the apparent discrepancy between single lipid binding and double density

We added a clarifying sentence regarding the double lipids. The density seen in the published structure is of four lipid tails next to each other, which is what one would expect for a CDL. Since the CDL could not be resolved unambiguously, two phospholipids with two acyl chains each were modeled into the density instead. Our MS and MD data strongly suggests that the density stems from a single CDL.

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