Determinants for forming a supramolecular myelin-like proteolipid lattice

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1 Abstract

2 Myelin protein P2 is a peripheral membrane protein of the fatty acid binding protein family.
3 It functions in the formation and maintenance of the peripheral nerve myelin sheath, and
4 several P2 mutations causing human Charot-Marie-Tooth neuropathy have been reported.
5 Here, electron cryomicroscopy of myelin-like proteolipid multilayers revealed a
6 three-dimensionally ordered lattice of P2 molecules between stacked lipid bilayers,
7 visualizing its possible assembly at the myelin major dense line. A single layer of P2 is
8 inserted between two bilayers in a tight intermembrane space of ~3 nm, implying direct
9 interactions between P2 and two membrane surfaces. Further details on lateral protein
10 organization were revealed through X-ray diffraction from bicelles stacked by P2. Surface
11 mutagenesis of P2 coupled to structural and functional experiments revealed a role for both
12 the portal region and the opposite face of P2 in membrane interactions. Atomistic molecular
13 dynamics simulations of P2 on myelin-like and model membrane surfaces suggested that
14 Arg88 is an important residue for P2-membrane interactions, in addition to the helical lid
15 domain on the opposite face of the molecule. Negatively charged myelin lipid headgroups
16 anchor P2 stably on the bilayer surface. Membrane binding may be accompanied by opening
17 of the P2 β barrel structure and ligand exchange with the apposing lipid bilayer. Our results
18 provide an unprecedented view into an ordered, multilayered biomolecular membrane system
19 induced by the presence of a peripheral membrane protein from human myelin. This is an
20 important step towards deciphering the 3-dimensional assembly of a mature myelin sheath at
21 the molecular level.

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1 Introduction

2 A central question in myelin biology is the molecular mechanism of the tight packing of
3 dozens of apposing lipid bilayers into a mature, multilayered myelin sheath. A major role in
4 this process is played by myelin-specific proteins. The high degree of order within the myelin
5 sheath has been known since early experiments using X-ray diffraction ¹; however, the details
6 of the molecular assembly have remained enigmatic.

7 The spontaneous formation of lipid membrane multilayers is a common functional property
8 of different myelin-specific proteins, which are not genetically related. In peripheral nervous
9 system (PNS) myelin, the compact multilamellar membrane contains only a few proteins.
10 The intrinsically disordered myelin basic protein (MBP) is irreversibly embedded into a
11 single leaflet of the lipid bilayer ². The cytoplasmic domain of myelin protein zero (P0)
12 behaves much like MBP, although it embeds deeper into the membrane ³. Full-length P0
13 promotes membrane stacking through both extra- and intracellular interactions ³⁻⁶. Peripheral
14 myelin protein 22 (PMP22), another PNS integral membrane protein, forms myelin-like
15 assemblies ⁷, much like those observed with MBP and P0. P2 adheres to the cytoplasmic
16 leaflet of the bilayer and can be classified as a peripheral membrane protein ⁸.

17 Peripheral membrane proteins associate with cellular membranes *via* diverse mechanisms.
18 Membrane binding may be either irreversible, mediated by post-translational modifications
19 (palmitoylation, myristoylation, or prenylation), or reversible with variable binding affinities.
20 The specificity of protein-membrane interactions is affected by the physical properties of the
21 protein and the lipid bilayer, such as surface charge or membrane curvature. Many peripheral
22 membrane proteins utilize amphipathic helices or hydrophobic amino acids that penetrate into
23 the hydrophobic bilayer core to form stable interactions with membranes ⁹.

24 P2 is a Schwann cell-specific protein expressed in the PNS myelin of tetrapods ¹⁰.
25 Intriguingly, P2 is expressed in a mosaic fashion, not being present in all myelin sheaths ^{11,12}.
26 This small β -barrel protein belongs to the family of fatty acid binding proteins (FABPs). The
27 bound fatty acid is enclosed inside the β barrel by a lid formed by two adjacent α helices
28 ¹³⁻¹⁵; the opening of the β barrel may be of importance in fatty acid entry and egress ¹³. In
29 addition to fatty acid binding, P2 can transfer lipids from/to membranes using a collisional
30 transfer mechanism ¹⁶, as seen with several other FABPs ¹⁷⁻²¹. Besides fatty acids, P2 may
31 bind cholesterol ¹⁴, which is abundant in the myelin membrane and essential for myelination
32 ²². The tip of the α -helical lid is hydrophobic, while both ends of the β barrel present
33 positively charged surfaces ^{14,15}, and these properties are likely important, when P2 stacks
34 between two phospholipid bilayers.

1 Studies on P2-deficient mice revealed temporarily reduced motor nerve conduction velocity
2 and altered lipid composition in PNS myelin. However, the overall PNS myelin structure
3 remained normal ¹⁶. Further analyses on the mutant mice revealed that P2 has a role in
4 remyelination of an injured PNS ²³ and melanoma cell invasion ²⁴. Five Charcot-Marie-Tooth
5 1 (CMT1) disease point mutations in human P2 have been discovered ^{25–29}. Three
6 CMT1-associated P2 protein variants have been characterized at the molecular level, showing
7 altered fatty acid and lipid membrane binding properties. The most drastic CMT1 mutation,
8 T51P, also reduced the membrane stacking capability of P2 ³⁰. Overall, the stability of the
9 mutant proteins was decreased, even though crystal structures indicated only minor structural
10 changes compared to wild-type P2.

11 In the current study, we incorporated human P2 into a model membrane multilayer system
12 and visualized the myelin-like proteolipid structures using electron cryomicroscopy
13 (cryo-EM). P2-bicelle complexes were used for additional structural insights. We produced
14 mutated forms of P2 to establish determinants of lipid bilayer and fatty acid binding and used
15 atomistic molecular dynamics (MD) simulations to visualize the intimate interaction between
16 P2 and a myelin bilayer. We show the spontaneous formation of an ordered, crystal-like
17 lattice of P2 bound inside membrane multilayers and highlight factors that are important in
18 this process, which involves a conformational change in the protein. The results provide a
19 glimpse into the self-assembling properties of myelin proteins and lipid membranes, which
20 are likely to be crucial for correct myelination in the vertebrate nervous system.

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1 **Materials and methods**

2 **Protein production**

3 The expression and purification of human wild-type P2 (wtP2) was done as described ¹⁴.
 4 Mutagenesis and the expression and purification of P2 variants were described earlier ³¹.

5 **Electron cryomicroscopy and image processing**

6 0.6 mg/ml of purified wtP2 or the P38G variant were mixed with *E. coli* polar lipids (Avanti
 7 Polar Lipids) using a lipid:protein ratio of 2 (w/w), corresponding to a molar ratio of ~40, and
 8 incubated for 1-2 h at +23 °C. For grid preparation, samples were applied to
 9 glow-discharged, holey carbon grids (QUANTIFOIL R 1.2/1.3, R 2/2 or R 3.5/1). 3-μl
 10 samples were adsorbed for 1 min at +20 °C, 90% humidity. Grids were then blotted for 2 s
 11 and vitrified by plunging into liquid nitrogen-cooled liquid ethane using an FEI Vitrobot
 12 MK4 (Vitrobot, Maastricht Instruments). The frozen grids were imaged using FEI Titan
 13 Krios TEM operated at 300 keV. Images were recorded using a Gatan K2 Summit direct
 14 electron detector, in counting mode (0.2 sec/frame, 8 sec in total, 6-7 e/pix/sec). Movie
 15 frames were aligned with MotionCorr ³² and preprocessed by 2dx_automator ³³. The effective
 16 pixel size of the images was 1.3 Å/pixel. Particles were boxed with EMAN2 (Helixboxer) ³⁴
 17 and further processed by Spring ³⁵ with helical reconstruction. In total, 25 000 overlapping
 18 and CTF-corrected segments with the size of 240 x 240 pixels were used with a binning value
 19 of 2 to calculate 2D class averages.

20 **Structural analysis of P2-stacked bicelles**

21 0.5 mg/ml P2 was mixed with 0.5 mg/ml bicelles (phospholipid:dodecylphosphocholine
 22 (DPC) ratio 2.85, phospholipids 1:1 dimyristoylphosphatidylcholine
 23 (DMPC):dimyristoylphosphatidylglycerol (DMPG) and incubated for 1 h at room
 24 temperature. 4-μl samples were then pipetted onto glow-discharged carbon-coated copper
 25 grids before incubating for 1 min. Excess solution was removed with filter paper (Whatman),
 26 and the samples were washed with 4 drops of Milli-Q water. Samples were stained with two
 27 drops of 2% uranyl acetate for 12 s in each drop and air-dried. Transmission electron
 28 microscopy (TEM) was performed using a Jeol JEM-1230 (MedWOW) instrument.

29 To examine repetitive structures in turbid samples, 2, 10, and 20 μM P2 was mixed with 1, 2,
 30 or 3 mM bicelles in 20 mM HEPES (pH 7.5), 150 mM NaCl. Samples were prepared at
 31 ambient temperature right before the measurements and measured at +25 °C. Synchrotron
 32 SAXS data from the suspensions were collected at the PETRA III storage ring, DESY,
 33 Hamburg, Germany on the EMBL beamline P12 ³⁶. Data were processed and analysed using
 34 ATSAS ³⁷. Repeat distances in the sample were deduced from Bragg peak positions.

1 **Crystal structure determination**

2 All P2 variants were crystallized and X-ray diffraction data collected as described ³¹. Data
3 were processed with XDS ^{38,39}, and molecular replacement was done using Phaser ⁴⁰ using
4 human wtP2 (PDB code 2WUT) ¹⁴ as a search model. Structures were refined with
5 phenix.refine ⁴¹, and rebuilding was done in Coot ⁴². The structures were validated using
6 MolProbity ⁴³. The refined coordinates and structure factors were deposited at the PDB (see
7 Supplementary Table 1 for statistics and entry codes).

8 **Proteolipid vesicle aggregation**

9 5 μ M of each P2 mutant was mixed with DMPC:DMPG (1:1) vesicles in a buffer containing
10 10 mM HEPES (pH 7.4), 150 mM NaCl and incubated for 10 min at room temperature.
11 Lysozyme and BSA were used as negative controls. Turbidity was measured on a Tecan
12 Infinite M200 plate reader at 450 nm. The turbidity values were plotted as relative turbidity
13 compared to wtP2 from the same measurement series. For further characterization, the turbid
14 samples were centrifuged and the supernatant and pellet fractions analyzed by SDS-PAGE, in
15 order to detect cosedimentation of P2 with aggregated vesicles.

16 Turbidity was also studied with protein-bicelle complexes, using wtP2. For this purpose,
17 bicelles (phospholipid/detergent ratio 2.85) were prepared as above, but the phospholipid
18 composition was varied. Bicelles were at 5 mM and P2 at 33 μ M. wtP2 was simultaneously
19 used to examine the effect of phospholipid vesicle composition on protein-induced turbidity;
20 the lipid concentration was 0.5 mM. Turbidity was measured using a Tecan Spark 20M
21 microplate reader at +30 °C.

22 **Surface plasmon resonance**

23 Surface plasmon resonance (SPR) was used to determine the effect of mutations on P2 to the
24 binding of the protein to lipid monolayers using the Biacore T100 SPR instrument. Lipid
25 monolayers consisting of either DMPC or dimyristoyl phosphatidic acid (DMPA) were
26 immobilized on an HPA chip (GE Healthcare) according to the manufacturer's instructions.
27 P2 at 1.0 μ M was injected onto the chip at +25 °C using 10 mM HEPES (pH 7.4), 150 mM
28 NaCl as running buffer.

29 **Circular dichroism spectroscopy**

30 Circular dichroism (CD) spectra were measured in 10 mM sodium phosphate (pH 7.0) at a
31 protein concentration of 0.1 mg/ml, using quartz cuvettes with 0.1-cm pathlength and a Jasco
32 J-715 spectropolarimeter. Melting curves were measured using 0.2 mg/ml protein at 217 nm.
33 The temperature was increased 1 °C/min from +20 °C to +90 °C.

1 Synchrotron radiation CD (SRCD) measurements for selected variants were performed on the
2 CD1 beamline of the ASTRID storage ring at the ISA synchrotron (Aarhus, Denmark). Scans
3 from 280 to 165 nm were performed in 1-nm steps at +20 °C in H₂O. Three mutants with
4 large effects on membrane binding (L27D, R30Q, and P38G), were studied in a bicelle
5 environment (4:1 DMPC/DPC).

6 Bicelles and vesicles with varying phospholipid compositions were used for further SRCD
7 experiments with wtP2. 0.4 mg/ml wtP2 was mixed with 5 mM bicelles or 2.65 mM vesicles.
8 Spectra were recorded from 280 to 170 nm at +30 °C, using a 100-μm cuvette. These
9 experiments were performed on the AU-CD beamline of the ASTRID2 storage ring at the
10 ISA synchrotron (Aarhus, Denmark)

11 **Fluorescence spectroscopy**

12 The fluorescent fatty acid 11-dansylamino-undecanoic acid (DAUDA) was used to study
13 fatty acid binding by P2. DAUDA has been used to study ligand binding in FABPs before
14 ^{44,45}. DAUDA was dissolved in DMSO, and the final DMSO concentration in the samples
15 was 1%. 20 μM DAUDA was mixed with 0, 1, 5, or 10 μM protein. Samples were incubated
16 for 2 h at +23 °C. Fluorescence excitation at 280 nm was used and emission was recorded at
17 530 nm using a Tecan Infinite M200 plate reader.

18 The binding of wtP2 and the P38G mutant to cholesterol was studied using the
19 environment-sensitive fluorescent cholesterol analogue
20 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-chole-3β-ol
21 (22-NBD-cholesterol). The fluorescence intensity of 22-NBD-cholesterol increases and the
22 fluorescence emission maximum shifts, if the probe is moved to a non-polar environment.
23 100 μM 22-NBD-cholesterol stock solution was prepared in 100% ethanol, and the maximum
24 ethanol concentration in the sample was 2%. All experiments were carried out in 10 mM
25 HEPES (pH 7.5). 2 μM 22-NBD-cholesterol was incubated for 16 h at +23 °C with varying
26 amounts of P2. Fluorescence spectra were recorded on a Horiba Fluoromax-4 instrument,
27 using excitation at 473 nm and emission between 500-600 nm, with a bandwidth of 5 nm.

28 **Atomic scale molecular dynamics simulations**

29 Structures of wtP2 and P38G were prepared for the simulations essentially as described
30 elsewhere ⁴⁶. Briefly, the P2 structure with bound palmitate was taken from the PDB entry
31 4BVM ¹⁵ and converted to match an all-atom representation consistent with the CHARMM36
32 force field ⁴⁷, which was used for simulating the system components, unless mentioned
33 otherwise. The topology for wtP2 was directly obtained from this conversion. The P38G
34 mutation was made *in silico* and equilibrated in a water environment. Both protein-palmitate

1 complexes had a total charge of +10.

2 Lipid bilayers were constructed using the CHARMM-GUI membrane builder ⁴⁸. Two
3 different membrane systems were considered: a 1:1 DMPC:DMPG bilayer as a general
4 reference with a net negative surface charge, and a myelin bilayer mimicking the cytoplasmic
5 leaflet of the myelin membrane. The composition of the myelin-like bilayer was 44 mol-%
6 cholesterol, 27 mol-% palmitoylcholinephosphatidylethanolamine (POPE), 2 mol-%
7 phosphatidylinositol-4,5-bisphosphate (PIP₂), 11 mol-% palmitoylcholinephosphatidylcholine
8 (POPC), 13 mol-% palmitoylcholinephosphatidylserine (POPS), and 3 mol-% sphingomyelin
9 ⁴⁹. The bilayers were symmetrical, comprised of a total of 200 lipid molecules each.

10 Ten Cl⁻ ions were included to neutralize the total charge of each protein-palmitate complex.
11 The systems were solvated with a total of 15000 water molecules each, with 0.1 M KCl.
12 Water was modelled using the TIP3P model ⁵⁰. Additional counterions (90 K⁺ in the
13 DMPC:DMPG and 32 K⁺ in the myelin membrane system) were included to neutralize the
14 system total charge. The total system volume was approximately (7.5 x 7.5 x 12) nm³ for the
15 DMPC:DMPG and (6.5 x 6.5 x 13.5) nm³ for the myelin membrane systems. Periodic
16 boundary conditions were used to make the bilayer structure continuous.

17 Molecular dynamics (MD) simulations were carried out under NpT conditions. Temperature
18 coupling was performed with the velocity-rescale method ⁵¹, using separate thermostats for
19 the protein, the bilayer, and the solvent. Reference temperatures were set at 310 K, with
20 coupling time constants of 2.0 ps. Pressure coupling was done semi-isotropically with the
21 Parrinello-Rahman barostat ⁵², using reference pressures of 1.0 bar with coupling time
22 constants of 2.0 ps and compressibility constants of 4.5 x 10⁻⁵ bar⁻¹.

23 All bonds were constrained with the LINCS algorithm ⁵³. Cut-off radii of 1.0 nm were
24 introduced for the Coulombic and Lennard-Jones interactions, including the neighbour list.
25 Long-range electrostatics were calculated using the particle-mesh Ewald method ⁵⁴ with
26 cubic interpolation and a spacing of 0.16 nm for the Fourier grid.

27 The simulation systems were built by adding the protein structure near the bilayer and
28 solvating the system thereafter. After a short steepest-descent equilibration, the systems were
29 simulated long enough for the protein to spontaneously come into close contact with the
30 bilayer. This was used as the starting structure, after which the systems were simulated for 3
31 μs each. A total of 4 full simulations were run (one for each protein-membrane combination),
32 in addition to several shorter simulations on the P2 membrane attachment phase. The first
33 500 ns of each simulation were removed as an equilibration period, and the final 2.5 μs were
34 used for analyses. All simulations were conducted with GROMACS 4.6.7 ⁵⁵, using the

- 1 CHARMM36 all-atom representation and a time step of 2 fs, saving the trajectory
- 2 coordinates every 50 ps.
- 3
- 4

1 **Results**

2 While the molecular composition of compact myelin is relatively simple, the arrangement of
3 proteins within the membrane multilayers is to a large extent unknown. Here, we used the
4 peripheral membrane protein P2 from the PNS myelin major dense line as a model system to
5 study myelin-like membrane stack formation and structure. P2 interacts with lipid bilayers
6 with high affinity^{13,15,56–58}. We explored its membrane binding characteristics, determinants,
7 and dynamics more closely. The results provide further information on the molecular details
8 of the major dense line in PNS myelin, as well as on CMT disease mechanisms linked to
9 mutations in P2.

10 **Arrangement of P2 in multilayered membrane stacks**

11 P2 spontaneously binds lipid membranes together, as reflected by earlier studies using
12 turbidimetry, simulation, and X-ray diffraction^{15,57}. However, the molecular details of this
13 phenomenon and the resulting supramolecular structure have remained elusive. Cryo-EM was
14 used to follow membrane stacking and ordering of proteolipid components in multilayers
15 induced by P2.

16 P2 induced the formation of highly ordered lipid bilayer stacks, while without P2, only
17 unilamellar vesicles were observed (Figure 1A,B). The angle between two separating bilayers
18 at the edge of a tight apposition is consistently $<60^\circ$ (Figure 1C). Although P2 is only 15
19 kDa, it is visible in cryo-EM images as ordered rows of particles between two apposed
20 membranes. Based on the calculated 2D class averages (Figure 1D-G), P2 evidently stabilizes
21 the lipid membrane stacks and defines the spacing (~ 3.0 nm) between two bilayer surfaces.
22 This myelin-like spacing between two apposing lipid bilayers is constant throughout the
23 membrane stacks. Based on the P2 crystal structure^{14,15}, the longest diameter of P2 is 4.5 nm,
24 indicating that either some parts of the protein are buried within the bilayer, or P2 is turned
25 on its side on the membrane. The repeat distance in the multilayer, containing a 4.5-nm
26 bilayer and a single layer of P2 molecules, is 7.5 nm. This is shorter than the distance
27 measured in solution with X-ray diffraction under more hydrated conditions, and close to the
28 distance observed with MBP and the P0 cytoplasmic domain in diffraction experiments^{2,3,15}.
29 P2 molecules are located between the bilayers with a lateral spacing of 3.5 nm between
30 monomers (Figure 1F), indicating lattice-like order between membranes. This order extends
31 into neighbouring membrane layers, and P2 molecules between the bilayers are at least to
32 some extent in register between consecutive layers (Figure 1G).

33 Cryo-EM was similarly carried out with the "hyperactive" P38G variant⁴⁶ mixed with lipids
34 (Figure 1E). Neither the bilayer spacing nor protein-lipid organization altered in the presence

1 of the mutant. During sample preparation, P38G induced membrane aggregation/stacking
2 faster than wtP2, and the turbidity effect was visible within 2-3 min (not shown), in line with
3 earlier experiments ⁴⁶.

4 **3-dimensional order in P2-bicelle complexes**

5 In order to obtain additional structural insight into P2-membrane complexes, P2 was studied
6 in a bicelle environment. P2 induced turbidity in protein-bicelle suspensions, and EM
7 imaging revealed stacked arrangements of bicelles in these samples (Figure 2A). Thus, X-ray
8 diffraction was used to gain more information on repetitive structures. In addition to the
9 Bragg peaks originating from membrane stacking repeats of ~7-8 nm, additional diffraction
10 peaks were observed (Figure 2B,C) in samples with the highest lipid and protein
11 concentrations. The corresponding distances are close to those expected from a lattice-like
12 setup of P2 molecules between two membranes, as observed in cryo-EM. The distances can
13 be used to deduce a possible lateral organization of P2 molecules in the membrane plane
14 (Figure 2D).

15 The distances observed in the experiment changed as a function of P/L ratio. This behaviour
16 is similar, but not identical, to that observed for the P0 cytoplasmic domain, which caused
17 tighter membrane packing at high P/L ratios ³. For P2, both the lipid and protein
18 concentration affect the repeat distance in a concerted fashion (Figure 2C). The distances get
19 shorter when lipid concentration increases, indicating an overall increase in order and tighter
20 packing. On the other hand, at the same lipid concentration, shorter distances are observed
21 with higher protein amounts. Hence, the protein and lipid components synergistically
22 assemble into a compact, ordered, 3-dimensional proteolipid structure.

23 **Design of point mutants**

24 In order to elucidate structure-function relationships in P2, as a general model for a FABP
25 with a collisional mechanism and tight interaction with membranes, we used the crystal
26 structure of human P2 to design mutations that might affect membrane binding (Figure 3A).
27 The electrostatic surface of wtP2 shows two positively charged faces, at the helical lid
28 domain and the bottom of the barrel structure (Figure 3B). The mutations can be roughly
29 divided into three classes: those removing positive surface charge, those affecting the
30 hydrophobic surface of helix α_2 , and other mutations possibly affecting the portal region.

31 **Crystal structures of P2 variants**

32 For a high-resolution insight into the structure-function differences in the P2 variants, their
33 respective crystal structures were solved (Supplementary Table 1, Figure 3C). None of the
34 mutations affected folding or secondary structure elements in the crystal state. With respect

1 to this observation, it is important to note that the three studied CMT disease variants of P2
2 crystallized like wtP2, even though their stability and function were impaired³⁰. The RMS
3 deviations of the mutant structures compared to wtP2 vary between 0.08 and 0.36 Å, P38G
4 being the most divergent.

5 Prior to the current work, all crystal structures for wtP2 or mutant P2 contained a bound
6 ligand inside the β barrel. The P38G structure refined here is the first exception: its internal
7 cavity is clearly empty; no electron density for a bound fatty acid is present. This allows
8 comparing details between liganded and unliganded P2 (Figure 3D). In our earlier study, the
9 P38G mutant contained bound palmitate⁴⁶. In the unliganded crystal structure of P38G, the
10 amino acid side chains pointing inwards mainly retain their conformation. The main-chain
11 hydrogen bond between residue 38 and Leu10 also exists in both P38G structures. However,
12 helix α2 at the portal region has slightly shifted outwards from helix α1 in both chains; a
13 similar change is observed in the R30Q mutant, which could be linked to altered
14 membrane-binding properties (see below). In addition, P38G electron density is poor for
15 residues 33-37 at the end of helix α2 of chain B, supporting an increased flexibility/partial
16 unfolding of the portal region in the P38G mutant in the absence of bound ligand, as seen in
17 earlier simulations⁴⁶. Phe57, as well as the whole β4-β5 loop of chain A, has somewhat tilted
18 away from the α2 helix.

19 All P2 structures excluding P38G have a fatty acid, modelled as a mixture of palmitate or
20 *cis*-vaccinate in the atomic-resolution structures^{15,59}, bound inside the β barrel. The
21 conformation and position of the fatty acid is similar in most structures. In the K65Q mutant,
22 the conformation of the palmitate is different, and Phe57 points outwards in all four chains in
23 the asymmetric unit (Figure 3E). This supports the proposed role for Phe57 as a gatekeeper
24 residue in the FABP family^{13,60}.

25 All human P2 crystal structures published thus far have an anionic group bound in proximity
26 of the hinge region; the identity of the ligand depends on crystallization conditions and
27 crystal contacts. In wtP2, either chloride or citrate interacts with Thr56 and Lys37^{14,15}. P38G
28 and F57A contain chloride and sulfate, respectively^{13,46}. In the CMT-associated P2 mutant
29 structures, there is a malate located in the anion binding site³⁰. In line with these data, all P2
30 mutant structures solved here have an anionic group bound in the same pocket. These
31 observations lend further support to the hypothesis that this pocket may be involved in
32 recognizing phospholipid headgroups and initiating membrane binding and/or conformational
33 change¹⁵.

34 **Membrane binding and multilayer stacking**

1 SPR was used to follow binding of the P2 variants onto immobilized lipid membranes, made
2 of either DMPC or DMPA (Figure 4A). These membranes are net neutral and negatively
3 charged, respectively. While MBP essentially binds to lipids irreversibly on SPR ², P2
4 dissociates from the membrane rapidly ¹⁵, suggesting different membrane interaction kinetics
5 for the two proteins with overlapping function.

6 Four P2 mutants showed decreased binding to lipid membranes. One of these is L27D, which
7 affects Leu27 at the tip of the helical lid and reduces the hydrophobicity of the portal region.
8 The other three mutations with reduced binding affinity towards lipid membranes are found
9 in adjacent loops on the opposite face, at the bottom of the β barrel. All these mutations
10 (K45S, K65Q, and R88Q) affect surface residues and reduce the positive charge at the
11 bottom of the β barrel. The locations of these mutations suggest two membrane binding
12 surfaces on opposite faces of P2, in line with its packing between two bilayers *in vivo* and *in*
13 *vitro*.

14 While some mutations caused diminished binding to the membrane surface, P38G and R30Q
15 had increased levels of binding. These two mutations are located in the vicinity of the portal
16 region and the helical lid domain. The difference in membrane binding of R30Q and P38G
17 compared to wtP2 was more pronounced, when a DMPC membrane was studied.

18 Turbidimetry was used to assess the effectivity of P2 variants in aggregating DMPC:DMPG
19 vesicles (Figure 4B). When the turbid proteolipid suspensions were centrifuged and analyzed
20 by SDS-PAGE, P2 co-sedimented with aggregated vesicles, and the strong proteolipid
21 complex was only partially solubilized by SDS; P2 was present as a ladder of oligomeric
22 forms (Figure 4C). Again, P38G was the most effective variant, stacking vesicle membranes
23 more than wtP2. Some mutations caused diminished turbidity compared to wtP2. The clearest
24 of these were L27D and R88Q; the latter lies in the β 6- β 7 loop - in the middle of a large
25 positively charged surface patch at the bottom of the β barrel (Figure 4D). Hence, again,
26 residues important for both membrane binding and stacking can be found on both positively
27 charged faces of P2.

28 Another turbidimetric experiment was carried out to compare bicelles and vesicles in
29 wtP2-induced multilayer formation. Like vesicles, bicelles are stacked by P2 into large
30 structures causing turbidity (Figure 4E). Such ordered complexes could be a step towards
31 higher-resolution structure determination of myelin proteolipid complexes, due to *e.g.*
32 restrained particle size and geometry.

33 **Fatty acid and cholesterol binding**

34 Using the fluorescent fatty acid analogue DAUDA, we followed internal ligand binding to P2

1 variants (Figure 5A). The situation is complicated by the fact that tightly bound fatty acids
 2 co-purify with P2 from the expression host. Thus, a quantitative analysis was not performed,
 3 as increased binding could reflect either higher affinity or lower amounts of copurified
 4 ligand. However, the level of bound DAUDA should correlate with the opening of the portal
 5 region and/or the barrel, which is also required for removal of the bound fatty acid. Since
 6 bound fatty acid affects dynamics of P2^{13,46}, it is likely that some of the mutated variants
 7 have different affinities towards fatty acids. Most mutant variants showed slightly higher
 8 DAUDA signal than wtP2, and P38G was the strongest binder of all variants.

9 We previously proposed cholesterol binding by P2¹⁴, in addition to fatty acids. Cholesterol
 10 binding was tested using wtP2 and P38G. wtP2 induced a clear, concentration-dependent
 11 change in the fluorescence spectrum of the environment-sensitive probe 22-NBD-cholesterol;
 12 the fluorescence maximum shifted towards shorter wavelengths and its intensity increased
 13 (Figure 5B,C). The spectral changes were more pronounced with P38G, which has a more
 14 flexible portal region⁴⁶. The experiment shows that cholesterol, which is very abundant in
 15 myelin, could be a physiologically relevant ligand for P2. These assays together indicate that
 16 protein flexibility is important when P2 binds to its biological ligands.

17 **Folding and stability of point mutant variants**

18 CD spectroscopy was used to analyze the folding and stability of the P2 variants. While most
 19 mutations had little effect on thermal stability, P38G had two steps of unfolding, the first one
 20 appearing already at 50 °C and the second one only at >75 °C (Figure 6A). Another outlier
 21 was R30Q, which had a slightly lowered stability compared to wtP2. Interestingly, these two
 22 mutations causing changes in stability are those with enhanced membrane binding and
 23 stacking properties. In the crystal structures, they present minor conformational differences in
 24 their helical lid, compared to wtP2.

25 To elucidate the conformational changes induced by membrane binding, SRCD spectra for
 26 wtP2 and some divergent mutants were measured in the presence and absence of DMPC:DPC
 27 bicelles (Figure 6B). For wtP2, bicelle binding induced small changes in the SRCD spectrum.
 28 L27D exhibited less change in the spectrum in the presence of bicelles, supporting the
 29 reduced membrane binding of L27D observed in SPR and turbidity assays. On the other
 30 hand, P38G, having a higher propensity for membrane interactions, showed larger
 31 conformational changes in the bicelle environment. R30Q behaved much like P38G; both
 32 variants showed partial unfolding. These results highlight the importance of protein
 33 flexibility in membrane binding and indicate a role for the α -helical lid in P2-membrane
 34 interactions.

1 The bicelle system was used to deduce effects of lipid composition on wtP2 folding state, as
2 well as to compare to vesicles with the same lipid composition. SRCD spectra showed that in
3 vesicles, both 1:1 and 9:1 DMPC:DMPG gave the same conformational change of wtP2
4 compared to the protein in water (Figure 6C). In bicelles, however, wtP2 behaved differently,
5 in that very little change occurred in DMPC alone or in 9:1 DMPC:DMPG, and the spectrum
6 changed more with 1:1 and 4:1 DMPC:DMPG, to resemble the one measured with vesicles.
7 (Figure 6D). These differences with respect to lipid composition could be related to
8 membrane curvature.

9 **Atomistic simulations on P2-membrane interactions**

10 To combine aspects of high-resolution structural data and membrane binding, we studied the
11 interactions of wild-type and P38G P2 with membrane surfaces using atomistic MD
12 simulations (Figure 7). Two membrane systems were built: a 1:1 mixture of DMPC:DMPG,
13 which corresponds to compositions often used in the laboratory, and a myelin-like membrane
14 based on literature values⁴⁹.

15 During the attachment of wtP2 onto the membrane surface, a similar orientation was always
16 observed: this involved the positively charged surface close to the bottom of the β barrel.
17 Arg88 is a central residue in initial P2-membrane interactions. While it was expected that
18 initial membrane binding would involve the portal region and the helical lid, this orientation,
19 with the bottom face of the β barrel first approaching the membrane, is reproducible. The
20 protein was further turned on its side in this arrangement in the myelin lipid composition,
21 indicating that the rows of P2 molecules observed in cryo-EM images do not embed deep into
22 the bilayers. The 3-nm spacing between membranes can accommodate one layer of P2 in this
23 orientation.

24 A difference in orientation was observed between the DMPC:DMPG and myelin membranes;
25 P2 remains more upright and dynamic in DMPC:DMPG, while it falls rigidly on its side on
26 the myelin-like membrane (Figure 7C). Differences in wtP2 dynamics were additionally
27 observed between lipid compositions. The protein was more rigid when bound to the myelin
28 membrane (Figure 7A); on DMPC:DMPG, it had higher dynamics and hung on the
29 membrane with the Arg88 anchor (Figure 7C). During the simulation with the myelin-like
30 bilayer, the PIP₂ molecules within the myelin bilayer bound to the tip of the β 5- β 6 and β 7- β 8
31 loops, promoting opening of the β barrel, while Arg88 at the other end of the protein, in the
32 β 6- β 7 loop, interacted strongly with POPS head groups (Supplementary Figure 1, Figure 7D).
33 The PIP₂ binding site is formed of the side chains of Arg78, Lys79, and Arg96. These results
34 could reflect an important difference between a biological membrane composition and
35 simplistic membrane models.

1 The electrostatic interactions of wtP2 and P38G were very similar with the membrane lipids
 2 during the simulations (Supplementary Figure 1). The P38G variant similarly attached to the
 3 myelin-like membrane surface, being anchored sideways, and opened up even more than
 4 wtP2 (Figure 7B,D). The portal region, and the expected opening during ligand exchange ¹³,
 5 face upwards in this setting, and upon the approach of another membrane, they could closely
 6 interact with its surface.

7

8

1 Discussion

2 Myelin protein P2 is a unique member of the FABP family, able to stack lipid bilayers
3 together, in addition to being a member of the FABP subgroup carrying out collisional
4 transfer. Lipid membrane binding by P2 involves the hydrophobic tip of the helical lid,
5 electrostatic interactions, and dynamics of the portal region^{13,15,46}. Here, we have revealed
6 details of the assembly of the P2-membrane stacks and the surprising role of the bottom
7 region of the P2 β barrel in membrane binding. The data provide much-needed information
8 on the assembly of the myelin membrane at the molecular level.

9 *Structure of P2-induced proteolipid multilayers*

10 Our cryo-EM experiments illustrate an organized lattice-like supramolecular 3-dimensional
11 arrangement of P2-membrane stacks. Surprisingly, P2, a 15-kDa protein, which has
12 dimensions of 4.5 nm x 3.6 nm, is visible between the lipid bilayers as a lateral network. Both
13 the cryo-EM images and calculated 2D class averages of P2-membrane stacks show a
14 constant distance (3 nm) between the apposing lipid membranes and a repeat distance
15 (containing a single bilayer and intermembrane space) of 7.5 nm. Earlier, a repeat distance of
16 ~9 nm for P2-membrane stacks was measured by X-ray diffraction using DMPC:DMPG in
17 suspension¹⁵. Bragg peaks in X-ray diffraction experiments support the highly organized
18 arrangement of P2-membrane stacks seen in cryo-EM, and the conditions for preparing
19 cryo-EM samples, with less hydration, might be more relevant to myelin *in vivo*. Indeed,
20 using the bicelle model system, we measured repeat distances of 7.5 nm in stacks of bicelles
21 induced by P2, and the distance evolved as a function of protein and lipid concentration.
22 Thus, P2 may have a function in defining the membrane spacing in PNS compact myelin,
23 together with MBP and P0. All three of these proteins produce membrane stacks *in vitro*^{2,3},
24 with intermembrane spacing very close to that seen in the mature myelin major dense line.

25 The spacing between the neighboring P2 molecules between membrane bilayers is constant
26 (~3.5 nm), and there appears to be a relationship between the positioning of P2 molecules
27 between consecutive membrane layers. The results suggest the presence of a near-crystalline
28 lattice of P2 between membranes; this is also supported by our X-ray diffraction experiment
29 using stacked bicelles, in which - unlike earlier similar experiments using lipid vesicles - we
30 see new distances much shorter than those coming from bilayer stacking *per se*. As these
31 distances depend on protein concentration, they correspond to distances between proteins
32 arranged as a lateral layer between two membranes. Whether such packing occurs *in vivo*,
33 depends on the local P2 concentration in myelin as well as the presence and organization of
34 other highly abundant myelin proteins, such as P0 and MBP. The quantity of P2 has been
35 reported to vary between different regions of PNS as well as from nerve fiber to nerve fiber

1 ¹².

2 We recently reported, using similar cryo-EM approaches, the arrangement of the extracellular
3 domains of P0 as a zipper-like assembly between the membranes ³. The assembly of P2 at the
4 cytoplasmic face shown here completes the picture of PNS myelin molecular assembly.
5 Importantly, while P0 extracellular domains interact with each other as two layers between
6 membranes, only a single layer of P2 is observed, and each protein molecule must interact
7 with two cytoplasmic leaflets simultaneously. The details of this aspect were further
8 characterized here through mutagenesis, functional experiments, and high-end computer
9 simulations.

10 *Functional residues revealed by point mutations*

11 The unique ability of P2 to stack lipid membranes requires two membrane-binding sites on
12 opposite faces of the protein; P2 has two positively charged surfaces. Membrane binding
13 experiments for surface-mutated P2 gave information about crucial regions and mechanisms
14 of protein-membrane interaction. The L27D mutation at the tip of the α -helical portal region
15 reduces membrane stacking and binding, as well as diminishes the changes in CD spectrum
16 upon introducing membrane-mimetic bicelles. Thus, Leu27 may be inserted into the
17 hydrophobic core of a lipid bilayer. This insertion is presumably facilitated by a
18 conformational change in the portal region ¹⁵. In addition, other portal region mutations
19 (K21Q, K31Q), which remove a positive charge, also decreased membrane binding and
20 stacking. These residues probably interact with negatively charged lipid head groups and,
21 together with Leu27, form a membrane anchor of the P2 portal region. We earlier showed
22 that the L27D mutation impairs the formation of stacked membrane systems in a cell culture
23 system ¹⁵.

24 On the other hand, the removal of a positive charge at the opposite end of the β barrel
25 (mutations K45Q, K65S, R88Q and K112Q) caused reduced membrane binding and stacking.
26 In MD simulations, R88Q protrudes into the lipid membrane and forms tight interactions
27 with lipid head groups, especially PS in the myelin-like bilayer. However, there are no
28 hydrophobic residues at the bottom face of P2, and the barrel bottom interaction with the
29 lipid membrane is facilitated by electrostatic interactions. The bottom region of P2 is unlikely
30 to be deeply inserted into membranes, nor will it undergo large conformational changes.

31 An exception within all P2 mutants concerns P38G. In line with earlier data ⁶¹, it is more
32 active in most of the experiments, including membrane stacking as well as membrane, fatty
33 acid, and cholesterol binding assays. The P38G mutation, however, does not alter the
34 organization or repeat distance of the P2-membrane stacks in cryo-EM. In the crystal

1 structure of P38G, there is no fatty acid bound, and this mutant is more flexible and has
2 altered dynamics compared to wtP2⁶¹. In MD simulations, the fatty acid was observed nearly
3 escaping from the barrel¹³. The weak electron density of the portal region in the P38G
4 mutant crystal structure supports the idea of a flexible lid in this mutant, making it more
5 dynamic^{46,62} and prone to opening. The other mutation, R30Q, which increased the
6 flexibility of the portal region, causes smaller but similar effects on the activity of P2 in
7 several assays, confirming the importance of the dynamics of the portal region in the function
8 of P2. For other FABPs, the Arg residue corresponding to P2 Arg30 has been suggested to
9 attract negatively charged fatty acids^{63,64}; while this could be happening in P2 as well, the
10 R30Q mutation clearly has larger-scale effects on membrane interactions and local folding or
11 dynamics.

12 Phe57 is a conserved residue within the FABP family suggested to be a general gatekeeper
13 for ligand binding^{13,60}. It controls ligand entry into the β barrel and can flip between two
14 conformations^{13,65}. Phe57 points outwards in the K65Q crystal structure, and the fatty acid
15 shifts towards the opening cleft. It is unclear how a mutation located at the other end of the β
16 strand might induce the flipping of Phe57. However, the Phe57 flip may be one initial step in
17 P2 opening, which was observed in MD simulations and structural studies^{13,30}.

18 Arg88 appears central to the initial P2-membrane contact, functioning as an anchor. Within
19 the human FABP family, Arg88 is conserved in P2, but not other family members¹⁵. This
20 indicates its possible importance for the membrane stacking function, since other
21 collision-type FABPs, bind transiently to single membrane surfaces.

22 *Conformational changes and dynamics upon membrane binding*

23 The binding of P2 onto a myelin-like membrane is accompanied by a conformational change
24 opening the likely entry/egress site of the bound fatty acid. This change can be observed both
25 experimentally and in computer simulations. The change is similar to that observed in
26 solution for the CMT disease variants and in extended computer simulations of P2^{13,30,46}.
27 Similar conformational changes were observed for H-FABP during long simulations⁶³. The
28 bound ligand could be exchanged with the apposing membrane in a multilayer, when this
29 conformational change occurs.

30 Atomistic simulations of P2 on a membrane surface revealed different behaviour on a
31 simplistic model membrane compared to a myelin composition. Importantly, some of the
32 lipids concentrated on the myelin membrane formed specific interactions with P2 during the
33 long atomistic simulations, contributing to the conformational change. The presence of two
34 anchor points for P2 membrane binding enabled the membrane to contribute to P2 barrel

1 opening, unraveling the bound ligand. The negatively charged lipids, PS and PIP₂, might also
2 affect other myelin proteins in a specific fashion, and further experiments will be required to
3 grasp the full scope of intertwined interactions between myelin proteins and specific lipids. A
4 model combining current data on P2 bound to the cytoplasmic leaflet of myelin is shown in
5 Figure 8.

6 Upon the formation of a P2-membrane complex, the dynamics of both the protein and lipid
7 components are altered. When bound to P2, the dynamics of the lipid membrane are
8 decreased⁵⁶, while P2 becomes extremely heat-stable when bound to membranes¹⁵. These
9 observations are likely to be linked to the synergistic effects of P2 and the lipids in the
10 tightening of the 3D molecular assembly, as shown by X-ray diffraction from
11 myelin-mimicking bicelle complexes. Furthermore, they are in line with the decreased
12 dynamics of P2 on a myelin-like membrane in the simulations.

13 **Concluding remarks**

14 We have shown that, similarly to P0, MBP, and PMP22, myelin protein P2 is able to
15 spontaneously induce the formation of myelin-like membrane multilayers. We have for the
16 first time visualized the arrangement of P2 between membranes, providing an unprecedented
17 view into the structure of the major dense line in peripheral nerves. Furthermore, our
18 observations provide a lipid composition-dependent mechanism for the opening of the P2
19 structure for ligand entry and egress; in the case of a multilayered membrane, the ligand
20 could be exchanged with the apposing membrane. How myelin proteins act together in
21 forming native myelin multilayers through interactions at both extracellular and intracellular
22 surfaces of the bilayer is a major question in myelin biology; the tools and materials exist for
23 solving this question in the coming years.

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10

Author contributions

S.R., M.L., I.V., H.S., and P.K. conceived of the study. S.R., O.C.K., J.K., T.N., M.L., A.R., V.P.D., and P.K. carried out the experiments. All authors contributed to the interpretation of the results. S.R. and P.K. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

16

1 **Figure legends**

2 **Figure 1. Electron cryomicroscopic analysis of lipid membrane stacking by P2.**

- 3 A. Cryo-EM image of *E. coli* polar lipid liposomes without protein. The images in A and
4 B are 480x480 nm in size.
- 5 B. The same liposomes in the presence of human P2 make myelin-like multilayered
6 stacks. Lipid-to-protein mass ratio is 2.0.
- 7 C. The angle between stacked membranes at the edges (pink circles) is nearly constant at
8 $\sim 60^\circ$.
- 9 D. 2D class averages of a single P2-linked bilayer stack. Lipid headgroups and proteins
10 are black. The size of the box is 140x140 pixels (18x18 nm).
- 11 E. Averaged structures of stacked membranes with wild-type and P38G mutant human
12 P2.
- 13 F. The space between two membranes is enough to fit one layer of P2. The membrane
14 diameter is 4.5 nm, the space between membranes 3.0 nm, and the distance between
15 individual P2 molecules 3.5 nm. The crystal structure of a P2 monomer has been
16 fitted into the assembly.
- 17 G. Averaging of P2-stacked multilayers, including two layers in the analysis, indicates
18 the lattice-like arrangement of P2 throughout the myelin-like multilayer. Lipid
19 headgroups and proteins are white; scale bar, 5 nm.

21 **Figure 2. Insights into P2 structure between membranes from bicelle complexes.**

- 22 A. Negative staining EM micrograph of P2-stacked bicelles. Scale bar, 100 nm.
- 23 B. Bragg X-ray diffraction peaks from P2-stacked bicelles (black) and vesicles (red).
24 The corresponding repeat distances are marked.
- 25 C. Titration of protein and lipid concentration in the bicelle samples indicate shorter
26 distances and higher order when both protein and lipid concentrations increase.
- 27 D. A model of P2 arrangement on the plane of the membrane, based on the peak
28 positions in (B).

30 **Figure 3. Crystal structure analysis of selected P2 mutant variants.**

- 31 A. Stereo view of all mutants analyzed.
- 32 B. Surface electrostatics of human P2 reveal two positively charged faces at opposite
33 ends of the molecule.
- 34 C. View from the top on the C α traces of all P2 variant crystal structures indicates

- 1 flexibility of helix $\alpha 2$ and the $\beta 3$ - $\beta 4$ loop.
- 2 D. Conformational differences between liganded and unliganded P38G. P38G with
- 3 palmitate (PDB entry 4D6B ⁴⁶) (grey) is superimposed with the two monomers of
- 4 unliganded P38G (light and dark blue).
- 5 E. Partial opening of the portal in the K65Q variant (pink), superimposed on the wtP2
- 6 structure (PDB entry 4BVM ¹⁵).

7

8 **Figure 4. Assays on P2 variant conformation and function.**

- 9 A. DMPA (grey) and DMPC (white) membrane binding assays by SPR.
- 10 B. Turbidity assay with 1:1 DMPC:DMPG vesicles.
- 11 C. SDS-PAGE analysis of proteolipid pellets reveals SDS-resistant P2 multimers.
- 12 D. Surface electrostatics of the P2 bottom surface in wtP2 (left) and the R88Q mutant
- 13 (right).
- 14 E. Turbidity assay of wtP2 with DMPC:DMPG vesicles (red) and bicelles (black).

15

16 **Figure 5. Ligand binding by human P2.**

- 17 A. Binding of the fluorescent fatty acid DAUDA.
- 18 B. Binding of NBD-cholesterol by wtP2 (black) and P38G (red). Dashed line, ligand
- 19 alone; thin line, 10 μ M P2; thick line, 40 μ M P2.
- 20 C. Concentration dependence of fluorescence at 532 nm.

21

22 **Figure 6. P2 stability and folding.**

- 23 A. Melting curves for wtP2 and all studied mutants. The outliers are P38G (red) and
- 24 R30Q (blue). The thick black line represents wtP2.
- 25 B. Conformation of wtP2 and selected mutants in the presence (solid lines) and absence
- 26 (dotted lines) of DMPC:DPC bicelles.
- 27 C. Wild-type P2 in water (red dashed line), 9:1 DMPC:DMPG (thin black line), and 1:1
- 28 DMPC:DMPG (thick black line).
- 29 D. Wild-type P2 in water (red dashed line), and lipid:DPC bicelles containing DMPC
- 30 (black dashed line), 9:1 DMPC:DMPG (thin black line), 4:1 DMPC:DMPG (medium
- 31 black line), and 1:1 DMPC:DMPG (thick black line).

32

33 **Figure 7. MD simulations on P2 binding to lipid membrane surface.**

- 1 A. RMSF for wtP2 (black) and P38G (red) in 1:1 DMPC:DMPG (thin lines) and myelin
2 lipid composition (thick lines).
- 3 B. Distance of the $\beta 4$ - $\beta 5$ opening of the β barrel during the simulation. Colouring as in
4 (A).
- 5 C. Angle of the P2 β barrel axis with respect to the membrane surface. Note how both
6 wtP2 and P38G are rigidly anchored to the same orientation immediately after the
7 equilibration period.
- 8 D. Snapshots from the simulations. Left: wtP2 on DMPC:DMPG at 1135 ns. Middle:
9 wtP2 on myelin at 2060 ns. Right: P38G on myelin at 800 ns. Locations of the two
10 membrane anchors, Arg88 (blue arrowhead) and Arg78/Lys79/Arg96 (magenta
11 asterisks) are indicated.

12 **Figure 8. Model for P2-membrane interactions based on current data.** Shown is a
13 superposition of wtP2 crystal structure (blue) and the membrane-bound conformation of
14 P38G (orange). Upon membrane binding, Arg88 gets anchored by POPS molecules (red) and
15 the basic residues around the $\beta 5$ - $\beta 6$ loop interact strongly with PIP₂ (green). The opening of
16 the $\beta 5$ - $\beta 6$ flap exposes the fatty acid ligand (purple). Leu27, Phe57, and the anion binding
17 site are facing the apposing membrane surface in this setting.

18

19 **Supplementary Figure 1. Electrostatic interactions between key basic residues and**
20 **membrane lipids.** Green, PIP₂; black, POPS; red, cholesterol; blue, POPE; magenta, POPC;
21 orange, sphingomyelin. Note how Arg78, Lys79, and Arg96 have stable PIP₂ contacts, while
22 Arg88 has strong interactions with PS, and these interactions are observed for both wtP2 and
23 P38G.

24

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A

B

C

D

E

wtP2

P38G

F

4.5 3.0 4.5

G

A **B** **C** **D**

A **B** **C** **D** **E**

A **B** **C**

A **B** **C** **D**