Supporting Information

OPTIMIZED PHOSPHOLIPID BILAYER NANODISCS FACILITATE HIGH-RESOLUTION STRUCTURE DETERMINATION OF MEMBRANE PROTEINS

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Supporting Experimental Procedures

OmpX cloning, protein expression and purification

For the generation of truncated MSP protein variants, a pET28a expression vector (EMD) harboring the MSP1D1 gene sequence, a coding sequence for an N-terminal His_6-tag and a TEV cleavage site was used. Deletions were made by PCR and subsequent ligation of the generated linear blunt-ended product with T4 polynucleotide kinase and T4 Ligase (NEB). Expression and purification of all MSP proteins was done as previously described [1]. The OmpX gene from *E.coli* (SwissProt: P0A917) lacking its signal sequence (residues 1-24) was PCR-amplified from *E.coli* genomic DNA and inserted into a pET11a expression vector (EMD) via *Nde*I/*Bam*HI restriction sites. OmpX was produced as inclusion bodies in *E. coli* BL21 (DE3) cells. Induction of protein expression with 1mM IPTG was done at an OD_{600} of 0.7 and cells were incubated for 12-16 more hours at 37°C. For the production of [*U*-^{2}H,^{13}C, ^{15}N]-OmpX, bacteria were grown in M9 medium[2] supplemented with 1g/L [*U*-99%^{15}N]-NH_4Cl and 2g/L [*U*-99% ^{2}H,^{13}C]-glucose in 99% D_2O (Cambridge Isotope Labs). Inclusion body preparation was done as described[3] and the protein was finally solubilized in 6 M GuHCl, 50 mM Tris/HCl pH8.5, 100 mM NaCl, 5 mM EDTA. Refolding was done at room temperature by dropwise dilution of 5 mL 300 µM OmpX in 6 M GuHCl into 50 mL of 0.5% dodecylphosphocholine (DPC), 50 mM Tris/HCl pH8.5, 5 mM EDTA, 500 mM L-arginine followed by stirring for at least two more hours. Refolded protein was dialyzed twice over night against 5L of 20 mM Tris/HCl pH8, 5 mM EDTA and 20 mM sodium phosphate pH6.8, 100mM NaCl, 5 mM EDTA in a 3.5 kDa MWCO dialysis tubing, concentrated up to 1-2 mM and analyzed with 2D [^{15}N,^{1}H]-TROSY NMR experiments. By this procedure, the final DPC concentration is around 100-250 mM. Detergent concentration was determined by ^{1}H NMR spectroscopy.

Cell-free production of bacteriorhodopsin (bR)

Bacterioopsin (bO) with a N-terminal 10x-His tag was expressed using established cell-free expression protocols (Schwarz et al., 2007). Cell-free reactions were carried out in dialysis mode in the absence of detergents or lipids. The resulting protein pellet was resuspended in SDS buffer (20 mM SDS, 30 mM sodium phosphate pH 8) and loaded to a Ni-NTA agarose column. After washing with SDS buffer (6 column volumes), purified bO was eluted with SDS elution buffer (15 mM SDS, 40 mM sodium phosphate pH 4). Protein containing fractions were pooled. Typically the bO concentration of the pooled fraction was about 60 µM. The resulting protein-detergent solution was diluted 1:2 with refolding buffer (50 mM sodium phosphate, 150 mM NaCl, 200 µM retinal) and detergent-solubilized DMPC (50 mM Triton-X100, 25 mM DMPC). The DMPC concentration was calculated for each sample depending on the bO concentration to match a final bO:MSP1D1:DMPC ratio of 1:6:420 or a bO:MSP1D1ΔH5:DMPC ratio of 1:6:240 for the two different MSP constructs. Refolding of bO could be clearly followed by a characteristic color change of the sample. After 1 h, purified MSP protein with a cleaved of His-tag as well as Biobeads (Biorad SM2) to remove detergent were added (overnight at room temperature). Biobeads were removed and the resulting bR-nanodiscs were washed using a Ni-NTA agarose column (with detergent free buffer) to remove empty nanodiscs and residual detergent. The (imidazole) eluted bR-nanodiscs were concentrated and loaded to a gel-filtration column (Superdex S200, analytical or preparative scale). Representative fractions were collected and concentrated to obtain NMR samples. Isotope labeling of bR NMR samples during cell-free expression was achieved by replacing all (unlabeled) amino-acid except Trp, Gln and Asn with ^{2}H,^{15}N-ALGAL (Cambridge Isotope Labs) or ^{2}H,^{15}N,^{13}C-ALGAL amino acid mixtures (Sigma).

Nanodisc assembly

Nanodiscs were assembled according to established protocols [1,4]. Briefly, MSP protein and cholate-solubilized lipids (Dimyristoylphosphatidylcholine, DMPC: Dimyristoylphosphatidylglycine, DMPG=3:1) were incubated with OmpX in 20 mM Tris/HCl pH7.5, 100 mM NaCl, 5mM EDTA (MSP buffer) at a final cholate concentration of 20mM. The MSP to OmpX ratio was kept at 4:1 with 200 µM of MSPs. The optimal lipid to MSP ratio was 1:65, 1:40 and 1:20 for MSPD1, MSPD1ΔH4 or ΔH5 and MSP1D1ΔH4H5, respectively. The MSP:DMPC ratio was determined empirically by varying the lipid content to obtain a largely monodisperse SEC profile. After incubation at RT for one hour, 1 g of washed Biobeads SM-2 (Biorad) was added per mL of assembly mixture and the suspension was shaken at room temperature for 4-12 h. Finally, biobeads were removed by centrifugation and washed once with MSP buffer. The supernatants were pooled, concentrated and purified on a S200 size exclusion column equilibrated with 20 mM sodium phosphate pH6.5, 50 mM NaCl, 5mM EDTA. The main peak was pooled and concentrated using an Amicon centrifugal device of 30 kDa MWCO (Millipore). The final NMR sample consisted of 1 mM [*U*-98% ^{2}H,^{13}C,^{15}N]-

OmpX in MSP1D1ΔH5 nanodiscs with d_{54}-DMPC/d_{54}-DMPG (3:1) (FB reagents, Cambridge, MA) in gel filtration buffer supplemented with 5 %(v/v) D_2O and 0.01 % (w/w) NaN_3.

Analytical size exclusion chromatography (SEC)

SEC experiments were performed with an Äkta purifier system (GE healthcare) using an analytical Superdex S200 10/200 GL column of 24mL bed volume. The column was equilibrated in 20 mM Tris/HCl pH7.5, 100 mM NaCl, 0.5 mM EDTA, sample injection volume was 500 µL and flow rate was 0.5 mL/min.

Electron microscopy

Nanodiscs were diluted to a final concentration of 50 - 250 nM in 10 mM Tris-Cl, pH8, 100 mM NaCl and adsorbed to glow-discharged, carbon-coated EM grids. Samples were prepared by conventional negative staining with 0.75% (w/v) uranyl formate as described previously [5]. EM images were collected with a Philips CM10 electron microscope (Philips Electronics, Mahwah, NJ, USA) equipped with a tungsten filament and operated at an acceleration voltage of 100 kV. Images were recorded with a Gatan 1 × 1 k CCD camera (Gatan, Inc., Pleasanton, CA, USA) at ×52,000 and a defocus value of ~1.5 µm. Images were processed and analyzed with the program ImageJ [6].

NMR spectroscopy and structure calculation

NMR experiments were recorded on Bruker AvanceI or AvanceIII instruments operating at 500, 600, 750 and 800 MHz proton frequency equipped with cryogenic probes. TROSY-based triple resonance experiments[7] were recorded in a non-uniform sampled (NUS) manner[8] using Poisson-gap sampling schedules[9]. 3D-NUS spectra were reconstructed using the iterative soft threshold (IST) method[10] and processed with NMRPipe[11]. Spectral analysis was done with SPARKY. The program Talos+[12] was used for the prediction of backbone phi and psi angles based on chemical shift data. The program RCI[13] was utilized for the estimation of the generalized squared order parameter S^2 of backbone amide moieties based on chemical shift data. For NOESY experiments a mixing time of 200 ms was used. Structure calculations were done with Xplor-NIH[14] using standard protocols. The 20 structures with the lowest restraint violation energies were used to obtain structural statistics of the ensemble. Covalent geometry of the best-energy structure was analyzed with PROCHECK-NMR[15]. ^{15}N relaxation was measured with TRACT[16] experiments and ^{1}H T1 and T2 times were obtained with proton inversion recovery and cmpg experiments, respectively.

Molecular dynamics simulations

OmpX was inserted into a hexagonal box of 1,2-di-myristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayer using the CHARMM-GUI web server (http:/www.charmm-gui.org)[17] in presence of 0.15 M KCl. Equilibration of the system was done at 318 K in two phases with 3 cycles each. The force constants to fix the position of the protein and membrane were gradually reduced in each cycle. In the first phase, a timestep of 1 fs and a simulation time of 50 ps in each cycle were used whereas in the second phase a timestep of 2 fs and a simulation time of 200 ps for each cycle were used. Total equilibration time was 750 ps. MD simulations of 25 ns duration were carried out with the isothermal-isobaric ensembles at 318 K with the program NAMD[18] in the absence of a transmembrane potential. Long-range electrostatic interactions were described using the particle-mesh Ewald method[19]. A smoothing function was applied to truncate short-range electrostatic interactions. Analysis of the obtained trajectories was done with GROMACS[20].

Supporting Information References

(1) Ritchie, T. K.; Grinkova, Y. V.; Bayburt, T. H.; Denisov, I. G.; Zolnerciks, J. K.; Atkins, W. M.; Sligar, S. G. *Methods Enzymol* **2009**, *464*, 211.
(2) Sambrook, J.; Russell, D. W. *Molecular cloning : a laboratory manual* Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 2001.
(3) Fernandez, C.; Adeishvili, K.; Wuthrich, K. *Proc Natl Acad Sci U S A* **2001**, *98*, 2358.
(4) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. *J Am Chem Soc* **2004**, *126*, 3477.
(5) Ohi, M.; Li, Y.; Cheng, Y.; Walz, T. *Biol Proced Online* **2004**, *6*, 23.
(6) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. *Nat Methods* **2012**, *9*, 671.
(7) Salzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wuthrich, K. *Proc Natl Acad Sci U S A* **1998**, *95*, 13585.
(8) Hyberts, S. G.; Arthanari, H.; Wagner, G. *Top Curr Chem* **2012**, *316*, 125.
(9) Hyberts, S. G.; Takeuchi, K.; Wagner, G. *J Am Chem Soc* **2010**, *132*, 2145.
(10) Hyberts, S. G.; Milbradt, A. G.; Wagner, A. B.; Arthanari, H.; Wagner, G. *J Biomol NMR* **2012**, *52*, 315.
(11) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J Biomol NMR* **1995**, *6*, 277.
(12) Shen, Y.; Delaglio, F.; Cornilescu, G.; Bax, A. *J Biomol NMR* **2009**, *44*, 213.
(13) Berjanskii, M. V.; Wishart, D. S. *J Am Chem Soc* **2005**, *127*, 14970.
(14) Schwieters, C. D.; Kuszewski, J. J.; Tjandra, N.; Clore, G. M. *J Magn Reson* **2003**, *160*, 65.
(15) Laskowski, R. A.; Rullmannn, J. A.; MacArthur, M. W.; Kaptein, R.; Thornton, J. M. *J Biomol NMR* **1996**, *8*, 477.
(16) Lee, D.; Hilty, C.; Wider, G.; Wuthrich, K. *J Magn Reson* **2006**, *178*, 72.
(17) Jo, S.; Kim, T.; Iyer, V. G.; Im, W. *J Comput Chem* **2008**, *29*, 1859.
(18) Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. *J Comput Chem* **2005**, *26*, 1781.

(19) Darden, T.; York, D.; Pedersen, L. *J Chem Phys* **1993**, *98*, 10089.
(20) Van Der Spoel, D.; Lindahl, E.; Hess, B.; Groenhof, G.; Mark, A. E.; Berendsen, H. J. *J Comput Chem* **2005**, *26*, 1701.

Supporting Information Figures

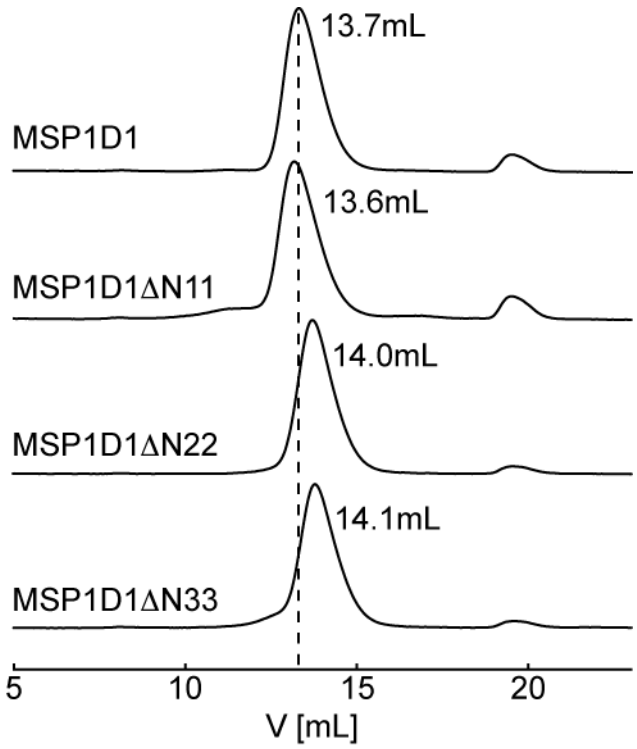

Fig. S1. Analysis of nanodisc sizes using N-terminally truncated MSP proteins. Analytical size exclusion chromatograms obtained with an analytical Superdex 200 column (GE healthcare) of DMPC nanodiscs formed with N-terminally truncated MSP variants. Buffer conditions were 20mM Tris/HCl pH7.5, 100mM NaCl, 0.5mM EDTA.

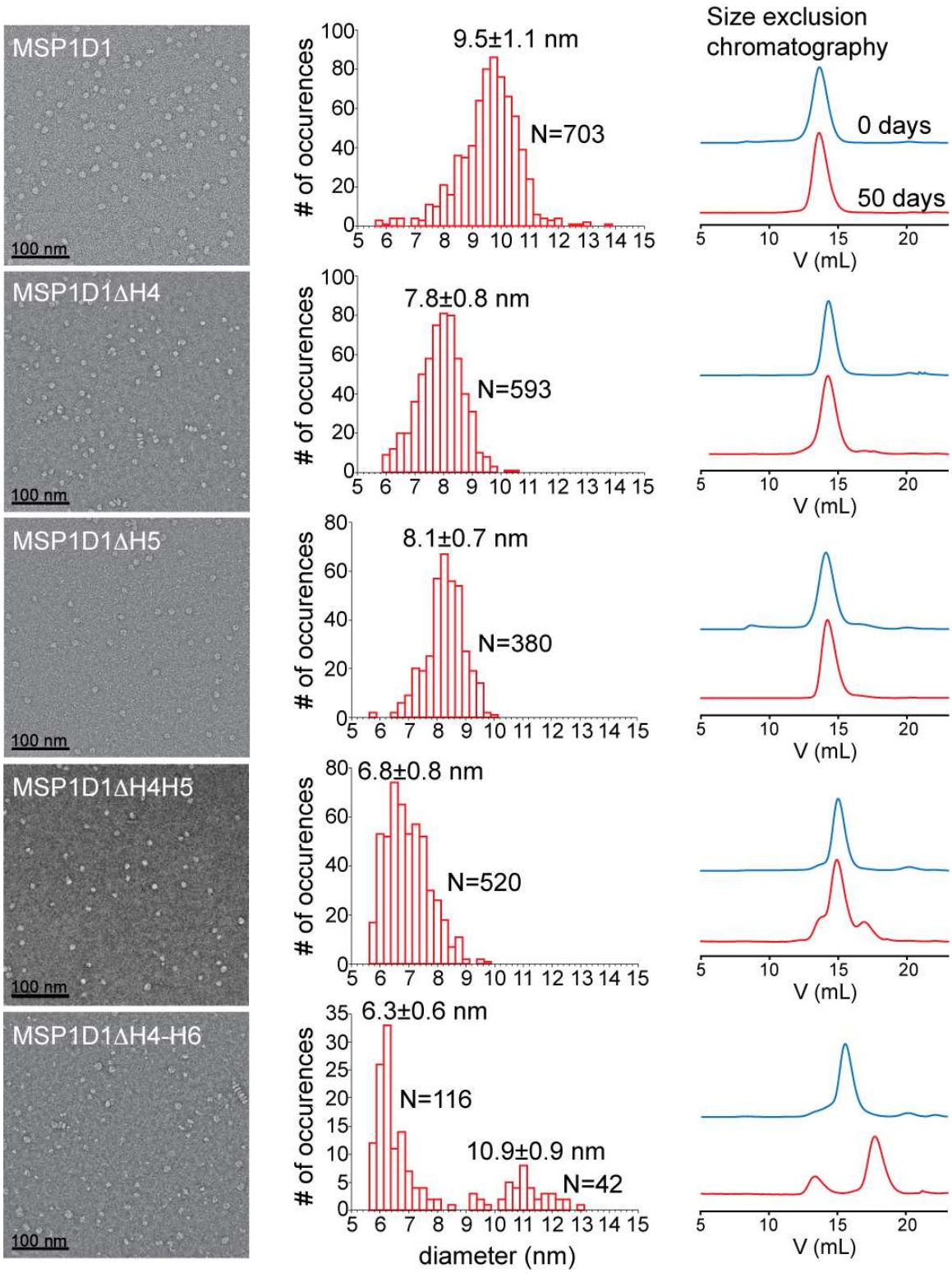

Fig. S2. Characterization of nanodisc size by electron microscopy (EM). Left: Representative negative stain EM images of nanodiscs assembled with (from top to bottom): MSP1D1wt, MSP1D1ΔH4, MSP1D1ΔH5, MSP1D1ΔH4H5, MSP1D1ΔH4-H6. Middle: nanodisc diameter distribution for each nanodisc size using a bin width of 0.25 nm. Mean values and standard deviations for each distribution and the number of analyzed particles (N) are indicated. Right: Size exclusion chromatograms of the analyzed nanodiscs right after assembly (top trace, blue) and after storage for 50 days at 4 ºC (lower trace, red). Buffer: 20 mM Tris/HCl pH7.5, 100 mM NaCl, 0.5 mM EDTA.

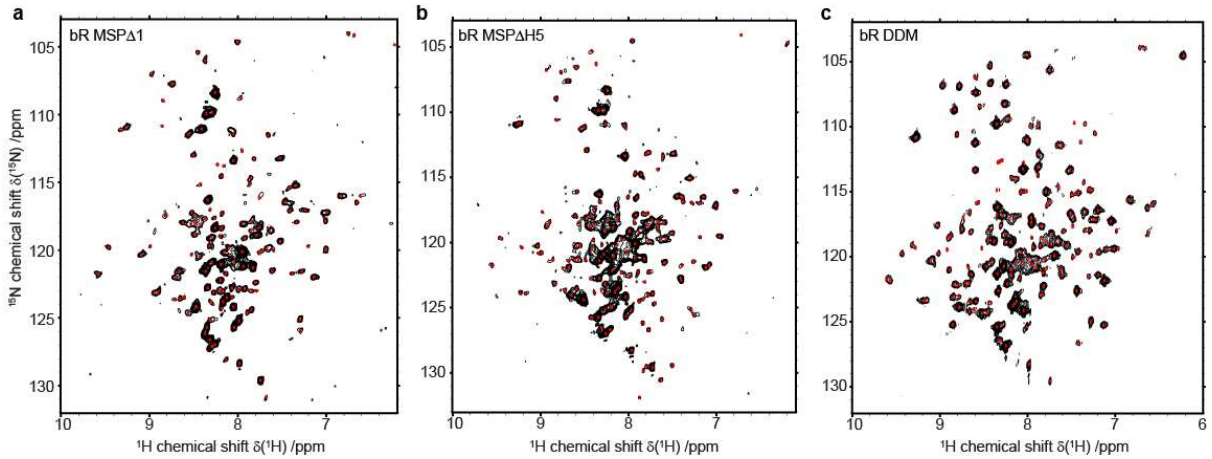

Fig. S3. Comparison of NMR spectral quality of bR in different membrane mimetic environments. 2D ^{1}H-^{15}N TROSY HSQC spectra of bR in DMPC nanodiscs formed using MSP1D1 (a) or MSP1D1ΔH5 (b) as well as in DDM micelles (c). All spectra were recorded at 40°C, a)+b) at 750MHz, c) at 800 MHz ^{1}H frequency. Using conservative data processing and peak picking (red peaks) the spectrum in a), b) and c) show 137, 154 and 184 resolved peaks, respectively. Note that bR concentration was different for the different samples (a) 150 µM, b) 80 µM, c) 400 µM) leading to a considerable better single-to-noise ratio of bR DDM. Nevertheless it can be seen that spectral quality is best when using the DDM micelle environment (as expected from the measured τc values (Fig. 3B). However, also in agreement with Fig. 3B, spectral quality of bR in DMPC nanodiscs improves by the usage of the MSP1D1ΔH5 when compared to the MSP1D1 construct.

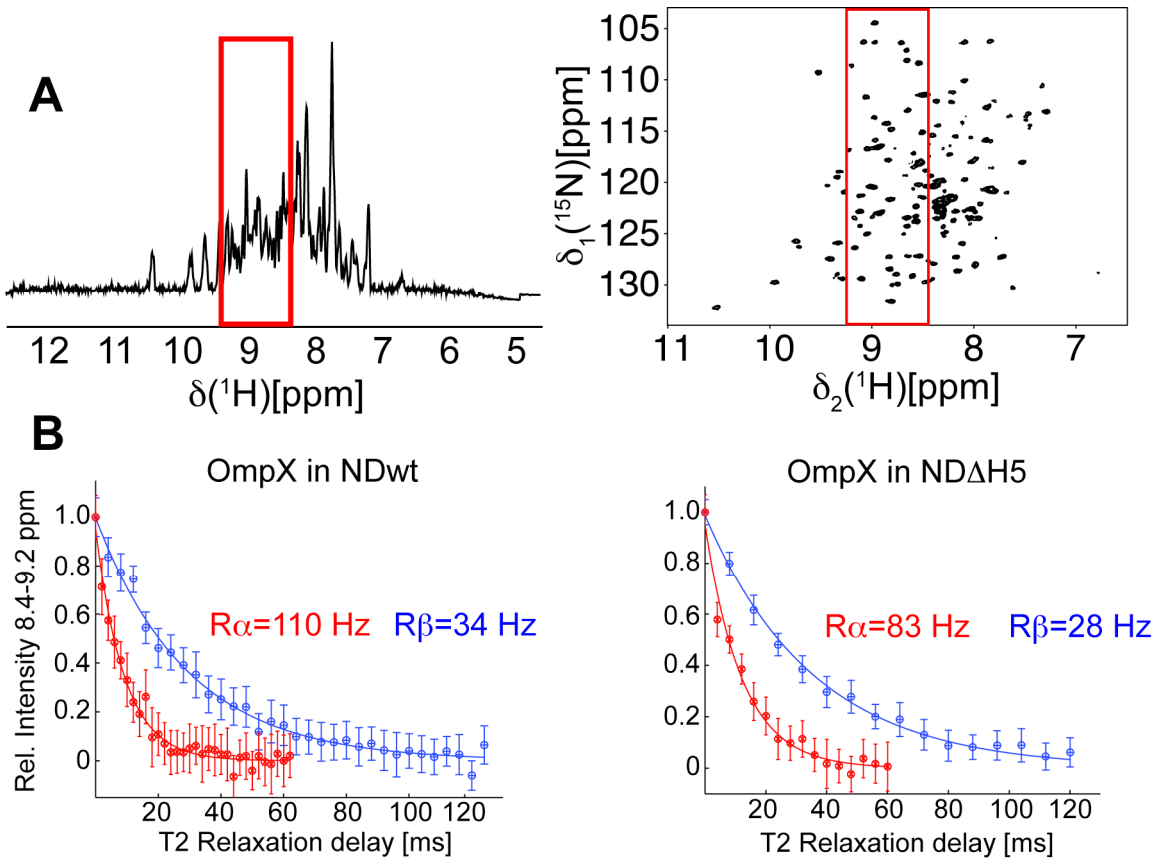

Fig. S4. T2 relaxation of OmpX in phospholipid nanodiscs. (A) 1D (left) and 2D (right) [^{15}N;^{1}H]TROSY spectra of OmpX in phospholipid nanodiscs at 45°C. The spectral region used for relaxation analysis is highlighted by the red box. (B) T2 relaxation curves of the amide TROSY (blue symbols) and the anti-TROSY (red symbols) component. Shown is the integral of signals within the boxed region in (A). Left: OmpX in NDwt; Right: OmpX in NDΔH5. Slower relaxation with the ΔH5 nanodisc as compared to the wt can be observed, indicative of faster overall tumbling and enhanced dynamics of the ΔH5 nanodiscs.

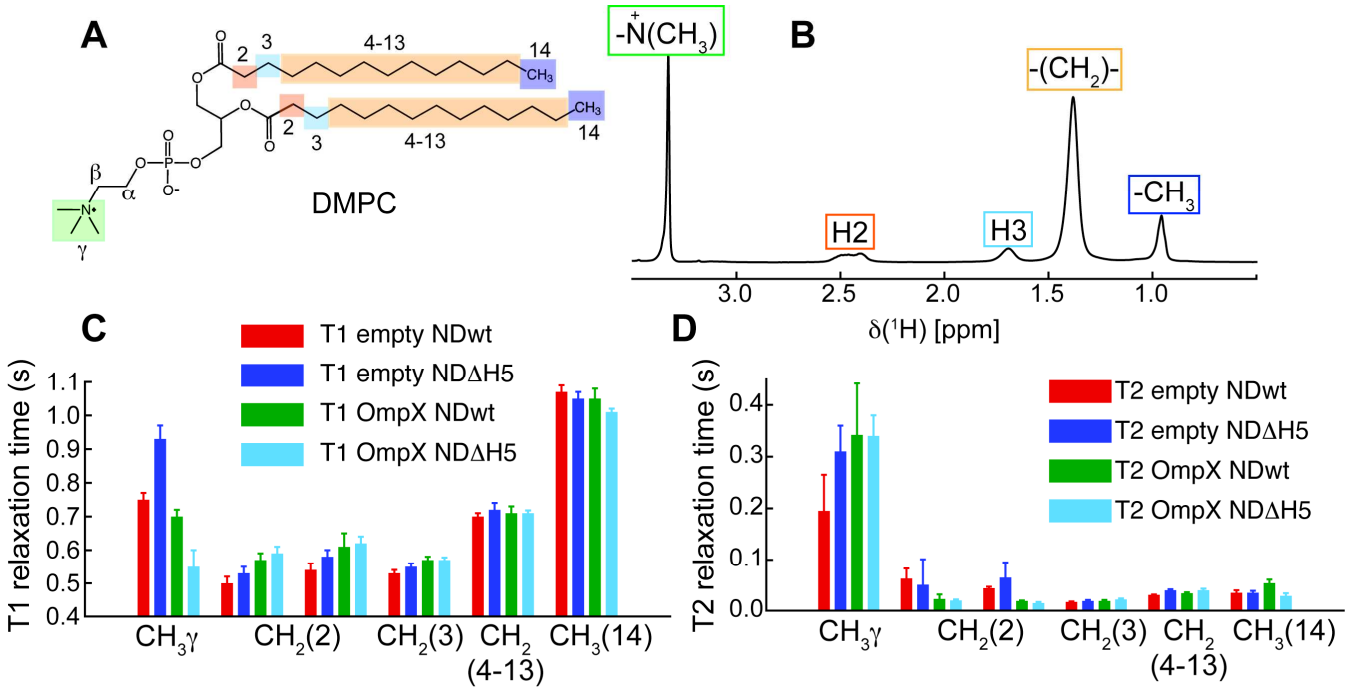

Fig. S5. Relaxation analysis of lipids in phospholipid nanodiscs. (A) Chemical structure of DMPC; assigned resonances in (B) are color-coded and numbered. (B) 1D [^{1}H] NMR spectrum of DMPC nanodiscs with the assigned chemical shifts labeled. (C) ^{1}H T1 relaxation times of DMPC lipid signals in various nanodisc preparations as labeled. (D) ^{1}H T2 relaxation times of DMPC lipid signals in various nanodisc preparations as labeled. T=318K, B_o=500MHz.

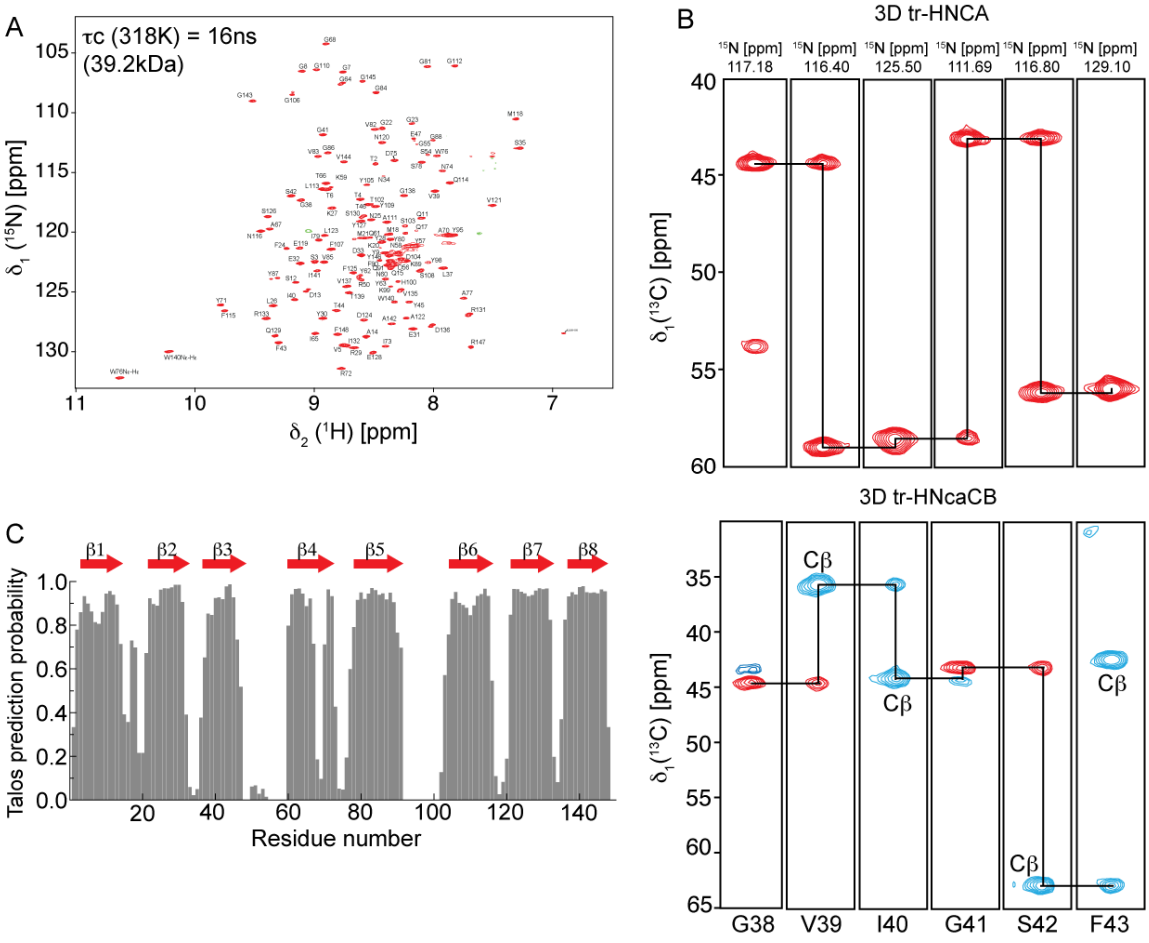

Fig. S6. NMR resonance assignment and secondary structure determination of OmpX in dodecylphosphocholine (DPC). (A) 2D-[^{15}N,^{1}H]-TROSY spectrum of 500 µM ^{2}H^{13}C^{15}N-labeled OmpX in 110 mM DPC at 318 K recorded at 500 MHz (B) 3D-TROSY-HNCA (top) and 3D-TROSY-HN(CA)CB (bottom) of OmpX in DPC micelles. (C) Talos+ secondary structure prediction probability for a β-sheet using the obtained chemical shift assignments.

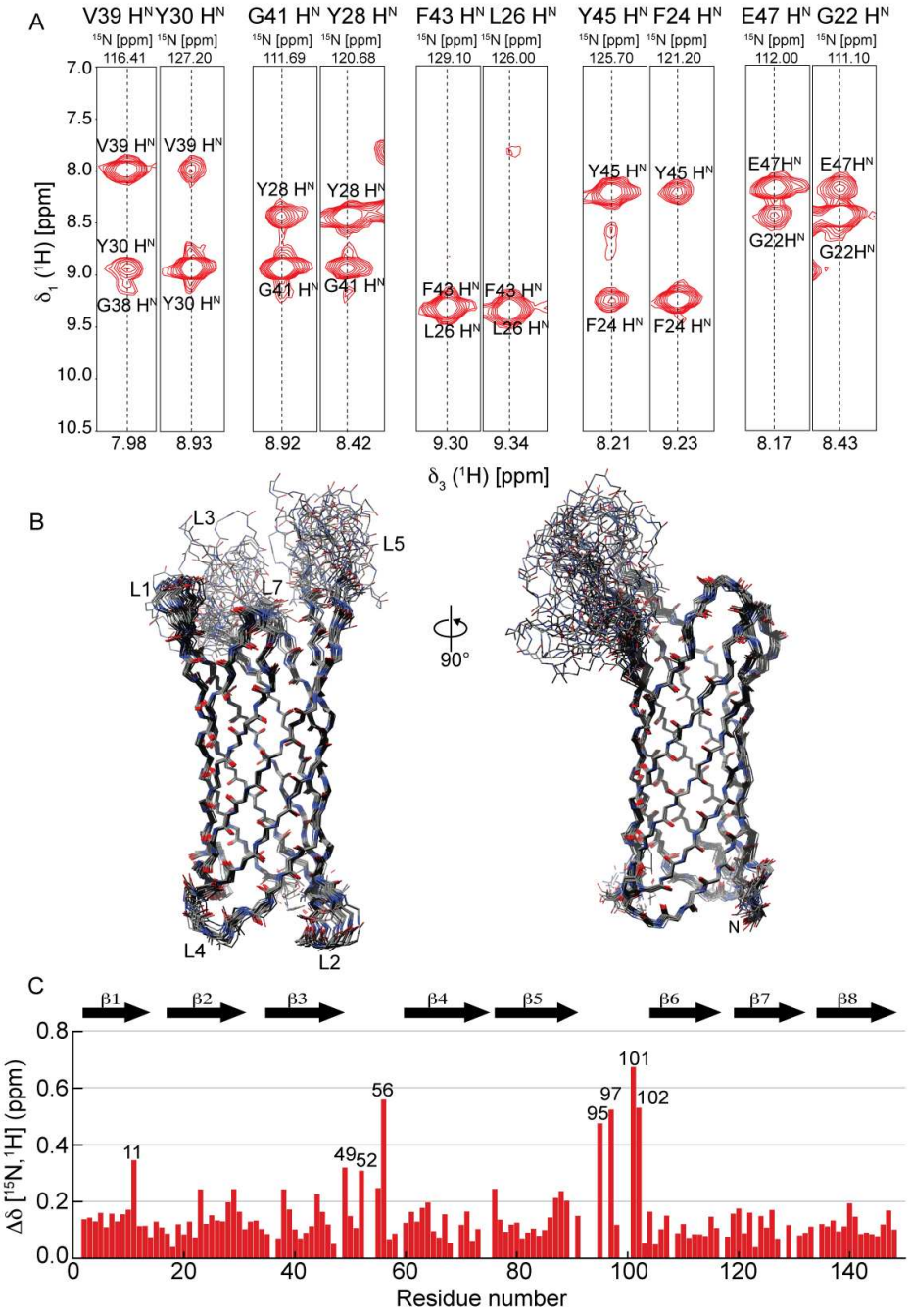

Fig. S7. Structure determination of OmpX in DPC micelles. (A) Representative regions of a 3D-^{15}N-edited-TROSY-[^{1}H,^{1}H]-NOESY spectrum of 500 µM ^{2}H^{13}C^{15}N-labeled OmpX in 110 mM DPC recorded at 500 MHz. (B) Ensemble of the 20 best-energy structures of OmpX in DPC having a backbone r.m.s.d. over residues in ordered secondary structure elements of 0.30 Å (see also Tab. 2). (C) Averaged ^{15}N, ^{1}H chemical shift differences of OmpX amino acid residues in DPC and in nanodiscs.