Two-state folding of the outer membrane protein X into a lipid bilayer membrane

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Experimental Procedures

Expression and purification of OmpX

Expression and purification of *E. coli* OmpX (24–171) was carried out as described previously.[1] In short, OmpX was expressed in *E. coli* BL21 DE3 cells, extracted from inclusion bodies, and purified by anion exchange chromatography (5 mL HiTrapQ HP column, GE Healthcare). Purified OmpX was solubilized in denaturing buffer containing 20 mM Tris-Cl, 2 mM EDTA, pH 7.8, and 6 M Gn-HCl. Protein concentration was determined by UV absorbance at 280 nm with an extinction coefficient of 34'840 $M^{-1}cm^{-1}$.

Preparation of small unilamellar vesicles

Aliquots of 10 mg PC10:0 (Avanti Polar Lipids) were dissolved in chloroform in a glass tube and subsequently dried to a thin film under a gentle stream of nitrogen gas and continuous shaking in a water bath. The lipid film was vacuum-desiccated overnight to ensure complete removal of chloroform. Vesicles were prepared by hydrating the lipid film with folding buffer (20 mM Tris-Cl buffer, pH 10.0, 2 mM EDTA and 600 mM arginine), and subsequent resuspension by pipetting and gentle vortexing. Small unilamellar vesicles (SUVs) were obtained from these vesicles by sonication for 10 min using microtip ultrasonifier at 50% pulse cycle. SUVs were equilibrated for a minimum of 30 minutes at 4 °C prior to be used in refolding assay. The particle size distribution of the SUVs was homogeneous with an average particle hydrodynamic diameter of 95 nm, as monitored by DLS measurements (Figure S3).

Gel-shift assay

From a denatured state in 6 M guanidinium hydrochloride (Gn-HCl), OmpX was rapidly diluted in PC10:0 vesicles in folding buffer with a lipid-to-protein molar ratio of 800. The final concentrations of OmpX and Gn-HCl in the refolding reaction were 5.5 µM and 33 mM respectively. At various time points, 5 µL of the refolding reaction was mixed with SDS loading dye and applied to a 4–20% SDS-PAGE (BioRad), followed by staining in Coomassie blue. Densitometry analysis was performed in ImageJ. The fraction of folded OmpX was calculated by dividing the folded band intensity by the sum of folded and unfolded band intensities. The fraction of folded OmpX in percentage was plotted against the refolding time and fitted to a single exponential function.

Real-time tryptophan fluorescence spectroscopy

Fluorescence emission spectra of OmpX folding into PC10:0 vesicles were measured in a JASCO spectrofluorimeter equipped with a Peltier temperature control. Gn-HCl denatured OmpX was rapidly diluted into PC10:0 vesicles in a lipid-to-protein molar ratio of 800 in refolding buffer and quickly transferred into fluorescence cuvette for measurements. The final concentrations of OmpX and Gn-HCl in the refolding reaction were 5.5 µM and 33 mM, respectively. Tryptophan emission spectra were recorded at 25 °C at various folding time points from 300 to 400 nm using an excitation wavelength of 290 nm. The excitation and emission band widths were set to 3 and 5 nm respectively. Spectra were acquired at a scanning speed of 200 nm/min with 2 nm increments. For kinetic analysis, the fluorescence intensity at the emission maximum of 340 nm was plotted against the folding time and fitted to a single exponential function. To observe the effect of LPR on folding kinetics, identical experiments were carried out at LPR of 100 resulting identical folding rate constants (Figure S5).

Proton/deuterium (H/D) exchange

OmpX folding into PC 10:0 vesicles under our folding conditions occurs on the minutes time scale, hence allowing H/D exchange experiments to be amenable using a custom-built pulsed quench flow apparatus (Figure S2). At the start of the experiment, 250 µL of 100 µM OmpX in 6 M Gn-HCl solution in D_2O and 2.5 mL PC10:0 vesicles in D_2O-folding buffer (20 mM Tris-Cl, pH 10.0, 2 mM EDTA, 600 mM arginine in D_2O) reside in syringe S1 and S2 respectively. The protein folding reaction is initiated at t = 0 by mixing the content of syringes S1 and S2 through mixing block I into syringe S3. Thereby, the Gn-HCl concentration is diluted to 0.55 M. Because the buffers of syringes S1 and S2 were prepared from D_2O, the unfolded OmpX in syringe S3 at t = 0 has close to 100% deuterons incorporated at all solvent-exchangeable positions, in particular in the backbone amide moieties. The sample in syringe S3 is then incubated for a variable folding time t = T and then mixed with syringe S4 containing 10 mL H_2O-folding buffer (20 mM Tris-Cl, pH 10.0, 2 mM EDTA, 600 mM arginine in H2O) through mixing block II into syringe S5. The sample in syringe S5 is then incubated for sufficient long time (typically overnight) to reach chemical equilibrium and subsequently analyzed by ESI-TOF mass spectrometry or solution NMR spectroscopy.[2]

Mass spectrometry

For each time point along the variable folding time T, folding of OmpX in PC10:0 SUVs was set up as described above with a molar lipid to protein ratio of 100:1. The folding reaction was run to completion and the solution was concentrated to 1 mL volume by ultrafiltration using 100 kDa cutoff membrane at room temperature followed by dialysis in a 10 kDa cutoff dialysis membrane for 6 h against 2 L of 5 mM ammonium acetate buffer, pH 6.3. 200 µL containing 20 µg protein was mixed with 1 mL cold acetone and kept at - 20 °C for 2 h. The protein precipitate was centrifuged at 13,200 *g* for 10 min at 4 °C. The supernatant was aspired, and the pellet was dried in a speedvac for 10 min. The protein pellet was resuspended with 50 µL DMSO containing 0.5 % (v/v) 1,3-dichloroacetic acid, vortexed for 30 s, and quick-spin for 10 s. The supernatant was applied by direct injection using a syringe pump and analysed with a Bruker Daltonics microTOF mass spectrometer. The spectrometer was calibrated at the onset of each experiment session with sodium-acetate clusters as mass standards.[3]

Solution NMR spectroscopy

250 µL of 6 M Gn-HCl denatured U-15N,2H-OmpX in D_2O were mixed with 2.5 mL PC10:0 SUVs prepared in D_2O-folding buffer, in a lipid to protein molar ratio of 100:1, using the manually operated custom-build set up as described above. The final 12.75 mL proteoliposomes were mixed with 3 mL 10% LDAO micelles and further vortexed, resulting in proteomicelles. The average hydrodynamic diameter of the proteomicelles were 5 nm as determined by DLS measurements (Figure S3). The solution was then concentrated to 500 µL and applied on a superdex 200 10/300 GL gel filtration column (GE Healthcare) pre-equilibrated with NMR buffer (20 mM sodium phosphate, pH 6.8, 1 mM EDTA, and 0.1% LDAO). Fractions containing OmpX were concentrated by ultrafiltration (3 kDa MW cutoff, Millipore) to a final volume of 200 µL. The final concentrations of each sample from various folding time points were identical in the NMR tube containing 5% D_2O. 2D [15N,1H]-TROSY NMR spectra were acquired at 30 °C in an NMR spectrometer with 900 MHz proton Larmor frequency and equipped with cryogenic triple-resonance probe. Spectra were processed in TopSpin 3.5 and analysed in CCPNmr.[4]

References

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Supplementary Figures

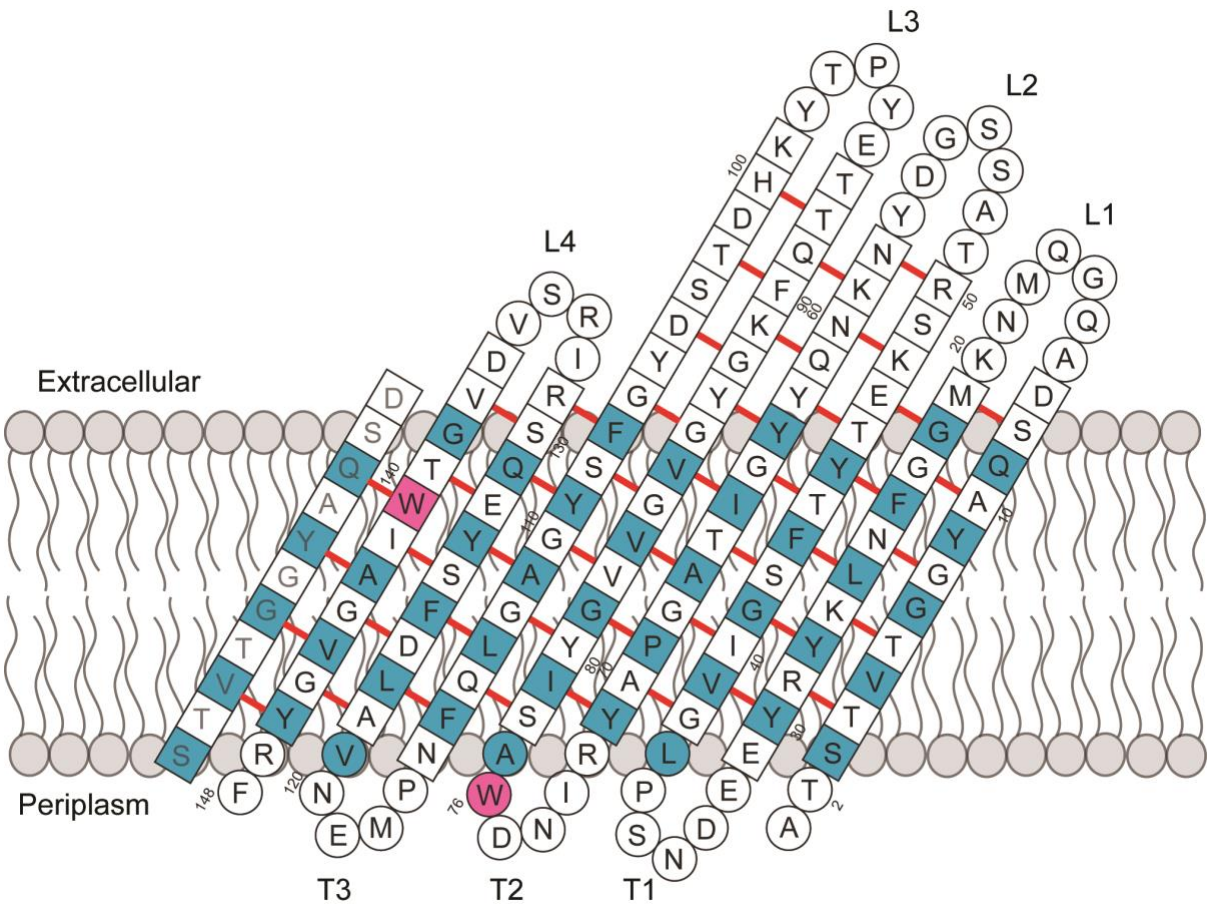

Figure S1: Transmembrane topology of *E. coli* OmpX. Residues forming transmembrane β-strands are indicated in rectangles, whereas residues of extracellular loops L1-L4 and periplasmic turns T1-T3 are indicated in circles. Red lines represent the interstrand backbone hydrogen bond pairs between corresponding residues. β-strand 1 is continued on left side of the figure to complete the hydrogen bond pairs with strand 8 and the barrel. Two tryptophans at position 76 and 140 shown in magenta were used for fluorescence spectroscopy.

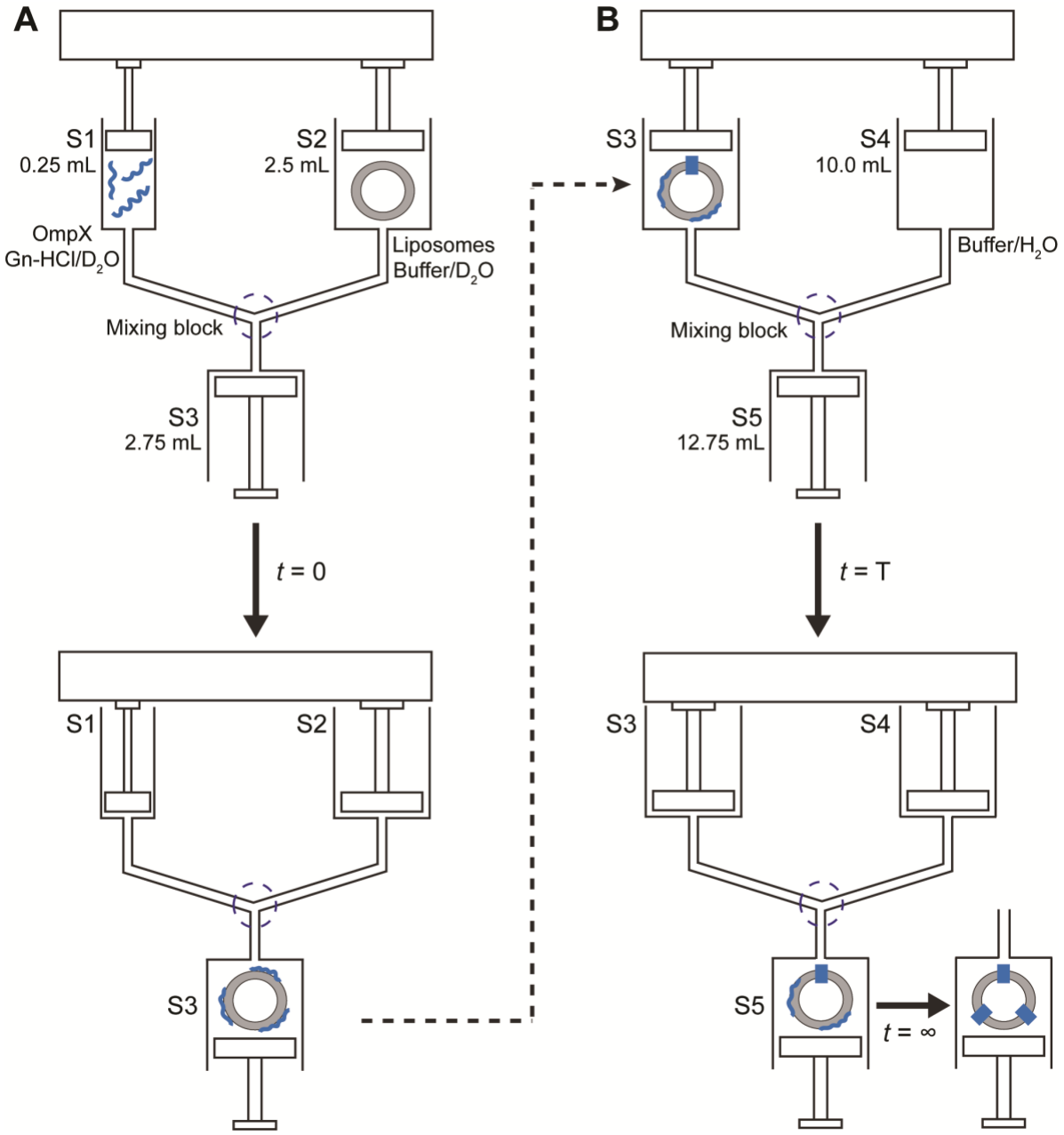

Figure S2: Schematic representation of the pulsed quenched flow mixing apparatus for H/D exchange studies of integral membrane protein folding into liposomes. (A) At the onset of the experiment, OmpX denatured in 6M Gn-HCl in D_2O and liposomes in D_2O-buffer reside in syringes S1 and S2, respectively. At t = 0, both solutions are mixed through mixing block I into syringe S3. The dilution of the Gn-HCl concentration to 0.55 M triggers the start of OmpX refolding. (B) After a variable refolding time t = T, the content of syringe S3 was mixed with H_2O-buffer from syringe S4 through mixing block II into syringe S5. Syringe S5 is then incubated for sufficient long time (typically overnight) to reach completion of the folding reaction. Subsequently, the incorporation of deuterium into the backbone amide positions is quantified by ESI-TOF mass spectrometry and NMR spectroscopy.

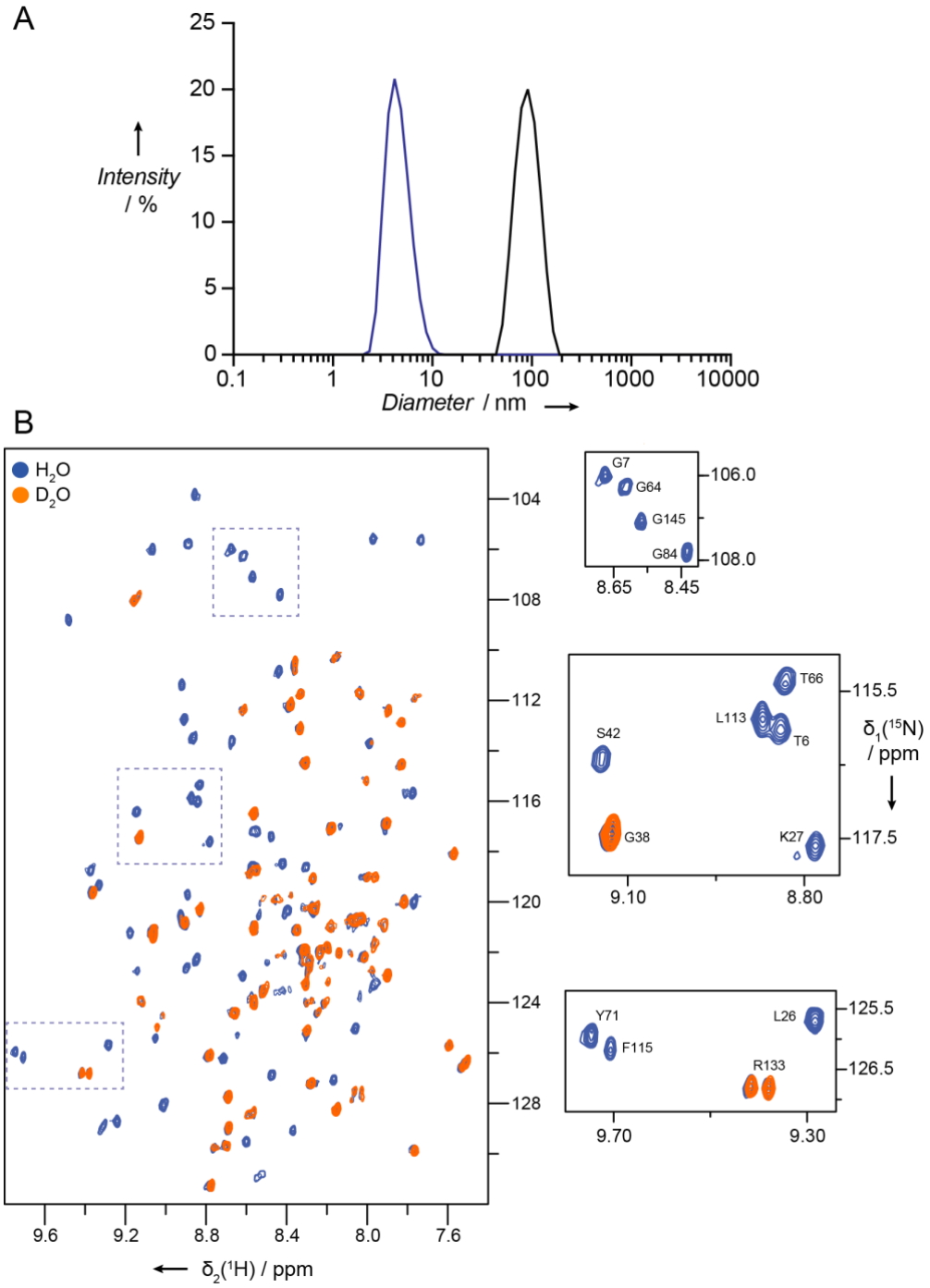

Figure S3: Transfer of OmpX from liposomes to detergent micelles. (A) Particle size distributions of PC10:0 liposomes (black) and OmpX in PC10:0 proteoliposomes after resolubilization with LDAO micelles (blue). Distributions were determined by dynamic light scattering with average diameters of 95 nm and 5 nm respectively. (B) 2D [^{15}N,^{1}H]-TROSY spectra of OmpX in LDAO micelles, after extraction a refolding reaction into liposomes. The refolding reaction took place in H_2O buffer (blue) or D_2O buffer (orange). Three selected spectral regions are shown enlarged on the right-hand side with sequence-specific resonance assignments indicated.

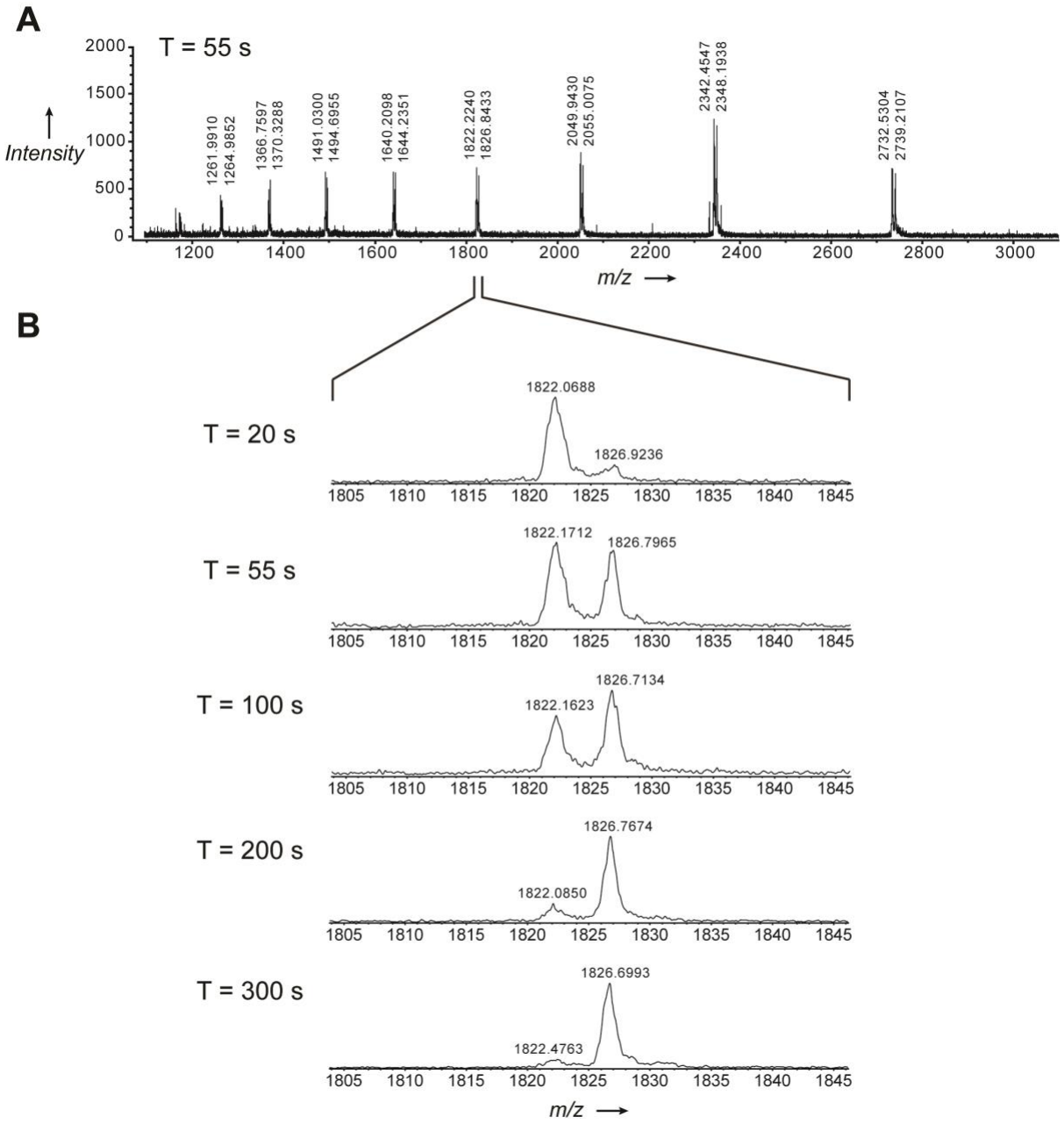

Figure S4: Representative ESI-TOF mass spectrum of OmpX. (A) A complete ESI-TOF mass spectrum of OmpX refolded in PC10:0 vesicles with H/D exchange time of T = 55 s. OmpX mass spectrum with various charged species were obtained between m/z of 1100 and 3000. (B) Section of the ESI TOF mass spectra of OmpX in PC10:0 vesicles at different H/D exchange folding time points. A bimodal population shift from protonated to deuterated species is observed without intermediate species.

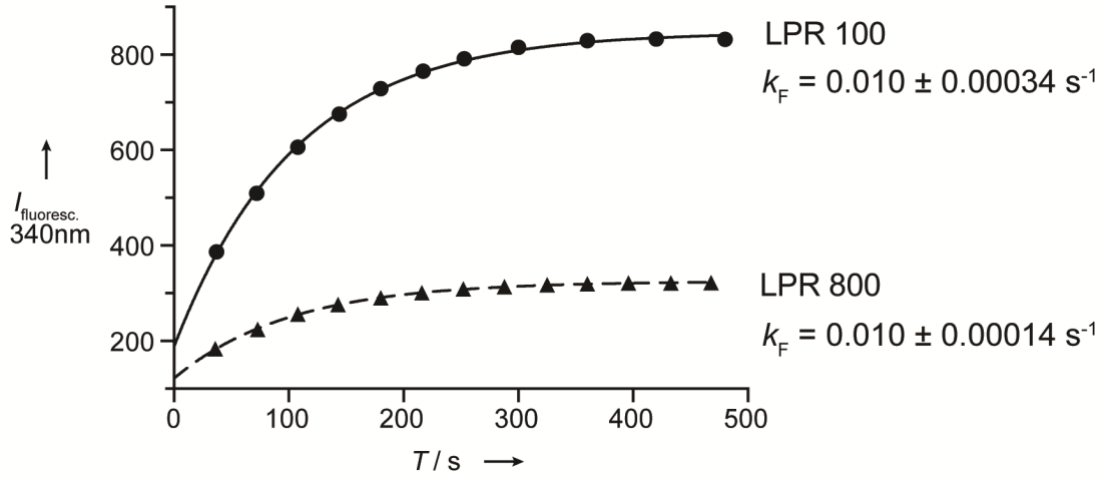

Figure S5: Folding kinetics of OmpX into PC10:0 vesicles are insensitive to the lipid-to-protein ratio. Fluorescence measurements were recorded at 25 °C on a JASCO spectrofluorimeter equipped with a Peltier temperature control. Spectra were recorded at an excitation wavelength of 290 nm and emission wavelength of 340 nm with respective band widths of 3 nm and 5 nm. Data were fitted to a single exponential.