Supporting Information

Detailed Experimental Methods

Chemicals. Dodecyl phosphocholine (DPC) and octyl β-glucoside (β-OG) were purchased from Anatrace-Affymetrix (Maumee, OH). 1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DiPhPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Dodecyl methanethiosulfonate (DdMTS) was purchased from Toronto Research Chemicals (Toronto, ON). ^{15}N-isoleucine, $(^{15}NH_4)_2SO_4$ and ^2H_2O were purchased from Cambridge Isotope Labs (Cambridge, MA). All other chemicals were from Sigma (St. Louis, MO).

Protein Preparation. Wild-type OmpG, quietOmpG and mutant OmpGs that carried one or two cysteines (Y22C, S58C, E101C, L141C, S182C, D224C, I226C, S266C and Y22C/S182C) were prepared and refolded in β-OG and DPC as previously described [1]. All refolded proteins were kept in a buffer containing 25 mM Bistris, 50 mM NaCl, 0.05% NaN_3, 0.1 mM EDTA, 5 mM DTT and 0.5% DPC at pH 6.3, flash-frozen, and stored at -80 ^oC.

For ^2H,^{15}N-labeling, cells were grown in a M9 minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl,4 g/L glucose, 1 mM $MgSO_4$, 100 µM $CaCl_2$) with water replaced by 99% ^2H_2O. ^{15}N-labeling was obtained by adding 1 g/L of $(^{15}NH_4)_2SO_4$ to the medium. To improve cell growth and protein expression, 5 ml of 10X ^2H,^{15}N-Bioxpress solution (Cambridge Isotope Labs, MA) was added to every 1 L of deuterated M9 minimal medium. This shortened the time for cell growth from three days to one day.

For selective ^{15}N-labeling of isoleucine, the plasmid encoding the appropriate mutant was transformed into chemically competent CT19 cells. The medium for ^{15}N-labeling of isoleucine was prepared as described.[1] Proteins were purified and refolding was completed as for unlabeled and uniformly labeled wild-type proteins.

Dodecylation. Refolded samples in either DPC or β-octyl-glucoside were thawed at room temperature, an aliquot of DTT was added to bring the final DTT concentration to 10 mM, and the sample was incubated at room temperature for 1-2 hrs. DTT was removed by passing the sample through a PD-10 desalting column (GE Healthcare, NJ) using degassed 25 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, 0.05% NaN_3 pH 7.0 buffer, containing 0.2% DPC. Fractions containing the protein were pooled and concentrated to a volume of 0.5 ml. The protein concentration was determined by NanoDrop (Thermo Scientific, MA) analysis, using an extinction coefficient of 85,000 M^{-1} cm^{-1}. A 10-fold molar excess of DdMTS solution was then mixed with the protein solution and

incubated at room temperature for 30 mins. A second batch of 10-fold molar excess DdMTS was added and the reaction mixture was rotated overnight at room temperature to complete the reaction. The reacted mixture was then purified by passage over a Superdex 200 size-exclusion column to remove excess DdMTS and trace amounts of mutant OmpG dimers using a 25 mM Bis-Tris buffer (25 mM Bis-Tris, 50 mM NaCl, 0.1 mM EDTA, 0.05% NaN_3 and 0.2% DPC, pH 6.3). The completeness of the dodecylation reaction was confirmed by MALDI-TOF mass spectrometry and NMR spectroscopy. Final protein concentrations were adjusted to 0.5 mg/ml for electrophysiological recordings or 0.5 mM for NMR experiments.

Disulfide Bond Accessibility by DTT. Labeled I226C was reconstituted into 10 mM DMPC liposomes by preparing nitroxide-labeled and dodecylated I226C samples in β-octyl-glucoside , followed by extensive dialysis to remove all detergent. Final protein samples were adjusted to 0.1 mM by mixing with 25 mM Hepes buffer (25 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, pH 7.0). Fresh DTT was added to the protein sample to bring the final concentration of DTT to 5 mM. 30 ul aliquots of each protein sample were taken at reaction times of 1, 5, 10, 20 and 60 minutes. A 30 ul protein sample without DTT was used for background subtraction. The reaction was quenched by mixing the sample with 270 ul isopropanol and the protein precipitate was immediately spun down at 13,000 rpm for 2 min. The protein pellet was washed once with 9:1 isopropanol:H_2O. The pellet was dried at room temperature for 5 min and then dissolved in 50 ul urea buffer (8 M urea, 10 mM Tris, 0.1 mM EDTA, pH 7.0). 5 ul of 10 mM Ellman reagent was mixed with the re-dissolved protein sample and the reaction mixture was incubated at room temperature for 5 min. The absorbances at wavelengths 412 nm and 280 nm, reflecting free sulfhydryls and protein, respectively, were measured using the UV-Vis mode in a Nanodrop 1000 (Thermo Scientific, MA) instrument. The intensity ratios A_{412}/A_{280} were plotted against reaction times to assess the exposure of disulfide bonds of the nitroxide-labeled or dodecylated I226C samples.

Single-Channel Current Recording in Planar Lipid Bilayer. Single-channel currents were recorded in planar lipid bilayers under an applied voltage of 50 mV at room temperature in 1 M KCl buffer and at pH 6.0 as previously described.[2] A black lipid membrane of DiPhPC was formed in a 100 μm aperture that connects two 2.5 ml chambers in a Teflon cell. The voltage and current amplification were controlled by an Axon Axopatch 200A amplifier (Molecular Devices, CA). Single-channel currents were recorded using the program Labman (Dr. G. Szabo, University of Virginia) at a resolution of 1 kHz. Data were processed using the program WinEDR (v3.3.4, University of Stratclyde, Glasgow, UK). Several seconds of traces of at least 5 independent bilayer preparations were recorded

for each sample to obtain sufficient statistics and to confirm reproducibility. The errors reported are the root-mean-square errors of these independent measurements.

NMR Experiments. $^{1}H,^{15}N$-TROSY spectra were acquired at 600 MHz on a Bruker Avance spectrometer using a standard pulse sequence.[3] 0.5 mM protein samples in 150 mM DPC were prepared in 25 mM Hepes, pH 7.0 buffer, containing 50 mM NaCl, 0.1 mM EDTA, 0.05% NaN_3. DTT was added to a final concentration of 5 mM to unreacted, free Cys samples. Spectra were processed with NMRPipe [4] and Sparky.[5] The chemical shift differences between dodecylated and unreacted samples were calculated using $\Delta\delta_{comp} = [\Delta\delta_{HN}^2+(\Delta\delta_N/6.25)^2]^{1/2}$, where $\Delta\delta_{HN}$ and $\Delta\delta_N$ are the chemical shift differences in the proton and nitrogen dimensions, respectively.[6]

Transverse Relaxation Time (T_2) Measurements. A pseudo3D pulse sequence [3a] was used to measure the ^{15}N T_2 relaxation times of both unreacted D224C and dodecylated D224C OmpG samples at pH 7.0. The length of a single CPMG loop composed by 16 spin echoes in ^{15}N dimension was set to 16.96 ms. Series of 12-20 spectra with different CPMG loops representing different relaxation delays in the ^{15}N dimension were acquired to extract the transverse relaxation rates by fitting peak intensities against relaxation delays to an exponential decay function. The assignment of I226 was obtained by comparing the ^{1}H-^{15}N TROSY spectra of selectively ^{15}N-isoleucine-labelled D224C. Correlation times τ_c were estimated using the simplified equation [7]

$$\tau_c \approx \frac{1}{4\pi\nu_N}\sqrt{6\frac{T_1}{T_2}-7}$$

and assuming T_1's of 3.2 s for immobilized and 1.2 s for free residues, respectively (B. Liang, T. Zhuang, and L. Tamm, unpublished results).

Relaxation Dispersion Experiments. ^{15}N relaxation dispersion experiments were performed with $^{2}H,^{15}N$-labeled samples at 800 MHz on a Bruker Avance spectrometer using the pulse sequence of Long et al. [8] A constant 50 ms delay was set for the CPMG pulse train with various numbers of spin echoes timed to result in CPMG frequencies ranging from 40 to 1400 Hz. Data points for each CPMG frequency were repeated at least once to assess experimental errors. To obtain exchange rates and intrinsic relaxation rates, the CPMG dispersion profiles were analyzed with the program NESSY using two- and three-site exchange models.[9]

References

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Supporting Figures

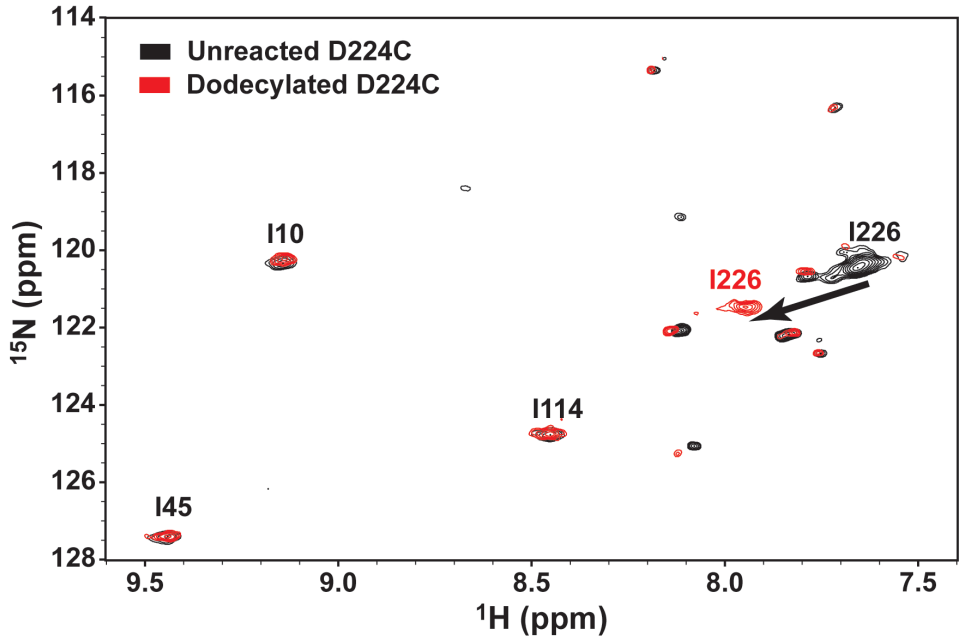

Figure S1. NMR spectra of the selectively ^{15}N-isoleucine-labeled D224C mutant of OmpG demonstrating the pinning of loop 6. The ^{1}H,^{15}N-TROSY cross peak of Ile226 in loop 6 moves from ~7.6 to ~8.0 ppm in the ^{1}H dimension from unreacted D224C (black) to dodecylated D224C (red). The other Ile cross-peaks remain unchanged. 0.5 mM protein, 150 mM DPC samples were prepared in 25 mM Hepes, pH 7.0 buffer, containing 50 mM NaCl, 0.1 mM EDTA, and 0.05% NaN_3. The unreacted D224C sample also contained 5 mM DTT. For the assignments of all isoleucines, see Zhuang et al 2013.[1]

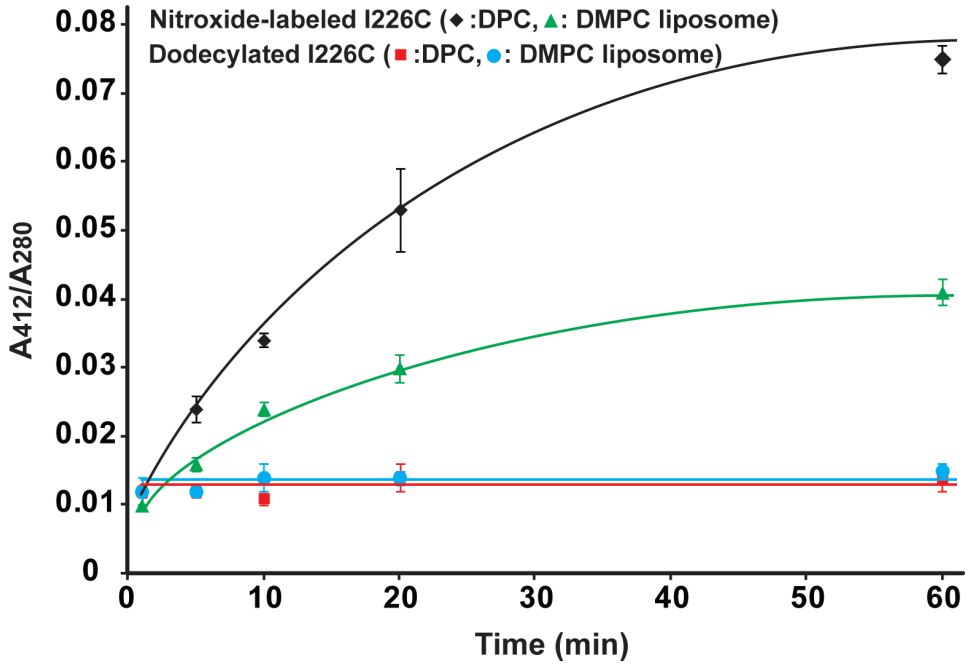

Figure S2. Time-course of disulfide-bond cleavage by DDT of nitroxide-labeled I226C in DPC micelles (black diamonds) or DMPC liposomes (green triangles) and dodecylated I226C in DPC micelles (red squares) or DMPC liposomes (blue circles). All protein samples were adjusted to a final protein concentration of 0.1 mM and treated with 5 mM fresh DTT at pH 7.0. The error bars represent the root-mean square deviations of two measurements.

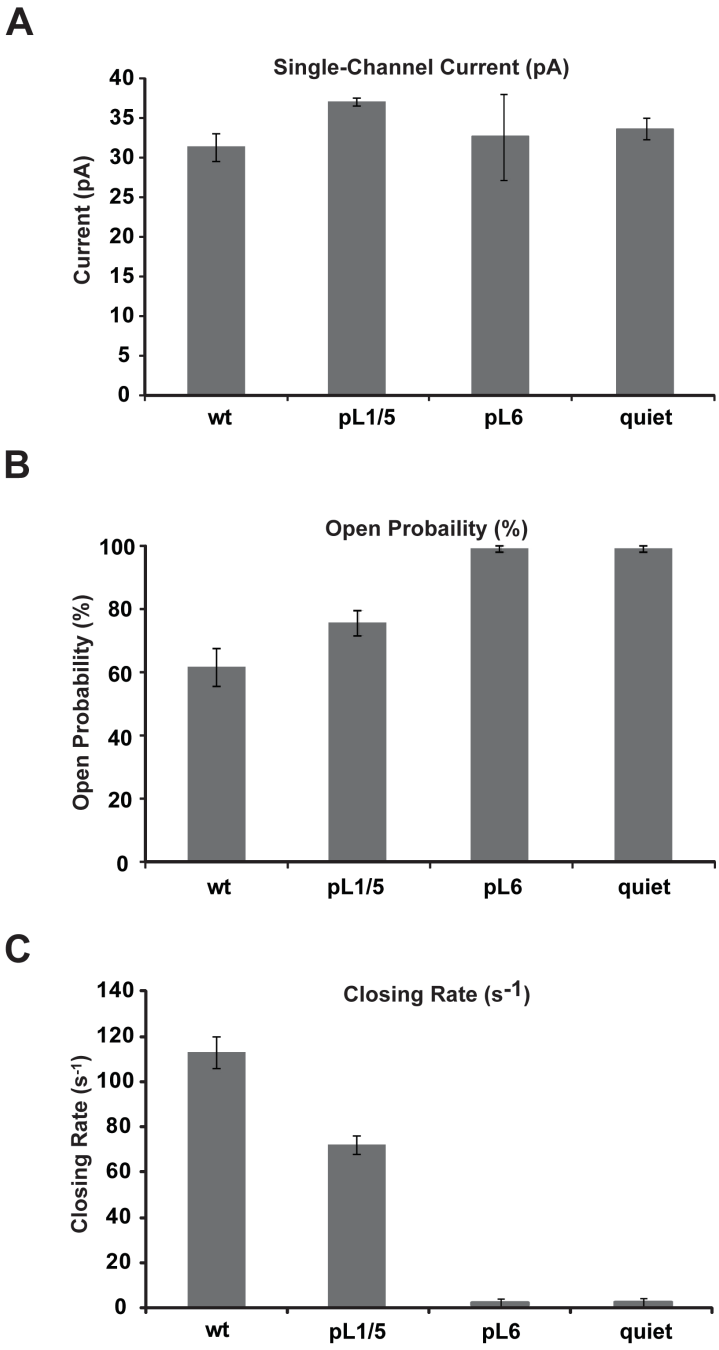

Figure S3. Comparison of the electrophysiological activities of singly (pL6) and doubly (pL1/5) loop-pinned and quiet mutants with the activity of the wild-type OmpG nanopore. Pinning of the main gating loop 6 creates a constitutively open pore similar to that of the quiet mutant with cross-linked strands 12 and 13 and deleted Asp215.[10] The double pinned pL1/5 mutant has an open probability and closing rate intermediate between wild-type and the constitutively open mutants. (**A**) Open current, (**B)** open probability, and (**C**) closing rates are compared.