Ligand induced conformational changes of a membrane transporter in *E. coli* cells observed with DEER/PELDOR

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Material and Methods

Cell growth

Mutations, culturing and spin labeling were performed as described before.[1] Briefly, the BtuB mutants 8C, 9C, 66C, 74C, 90C and 188C-399C in a pUC8 backbone were generated using Agilent Technologies (Santa Clara, CA) QuikChange Site Directed Mutagenesis Kit. The mutants were overexpressed in the *E. coli* strain RK5016 (*argH*, *btuB*, *metE*).[2] To avoid CN-Cbl repression of BtuB, cells were grown in minimal media for overnight. Media was supplemented with 3 m*M* MgSO4, 300 μ*M* $CaCl_2$, 0.24% w/v glucose, 150 μ*M* thiamine, and 0.01% w/v of Met and Arg and 100 μg/mL ampicillin.

Membrane isolation

The native outer membranes containing BtuB were isolated after selective removal of the inner membranes with sarkosyl detergent. Briefly, cells from 1 L culture were pelleted following overnight growth and suspended in 30 mL of 10 mM tris buffer (pH 7.5) containing 60 mM NaCl. The cells were lysed by French press for 2-3 times and the cell debris were removed by centrifugation for 20 minutes at 12,000 rpm using Sorval SS-34 rotor. The supernatant was collected and the cell envelop consisting of both the inner membrane and the outer membrane was pelleted by centrifugation at 100,000 x g for 1.5 hours. The pellet was suspended in the same buffer and the inner membrane was solubilized by adding 0.5% sarkosyl detergent. The outer membranes (OM) were pelleted with centrifugation at 200,000 x g for 1.5 hours. Following centrifugation, the pellet was suspended into 10 mL of the same buffer for spin-labeling.

Spin labeling

For spin-labeling, cells collected from 1 L culture was suspended in 30 mL of 50 mM Tris buffer (pH 7.5) containing 60 mM NaCl and 0.5% glucose. For spin labeling, MTSL (or MTSSL, 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate ,Toronto Research Chemicals, North York, ON, Canada) was added to 100 μM final concentration and incubated with shaking for 1 hour at room temperature. Following incubation cells were washed two times with the same buffer and suspended to $1x10^{11}$ cells/mL. The viability of the cells was checked by plating the serial dilutions on LB-agar plates. PELDOR samples were immediately prepared with addition of 15% d_8-glycerol and kept at -80^0C until measurements.

The outer membrane fraction as prepared above was spin-labelled by adding 40 μM MTSL and incubating at room temperature for 1 hour. Following incubation, the membranes were pelleted and washed several times until the free spins were completely removed and suspended into 500 μL final volume. As we reported previously, spin-labeling of outer membranes produces signals arising from non-specific labeling as well. However, those spins are far separated and do not give any distances in the measurable range.[1] Thus other than reducing the modulation depth and the absolute sensitivity, the non-specific labeling does not cause any problems for the PELDOR experiments.

MTSL reduction by *E. coli* cells

Cells collected from a 2 L overnight culture was suspended into 30 mL of 50 mM Tris buffer (pH 7.5) containing 60 mM NaCl and 0.5% glucose. MTSL was added to 150 μM final concentration and cells were incubated with shaking at room temperature. 1 mL samples were collected at periodic intervals, pelleted immediately and the supernatant was collected in a fresh tube. The spin concentration in the supernatant was determined from the double integral of the RT CW-EPR spectra using a calibration standard prepared from 4-amino TEMPO.

RT-continuous-wave EPR measurements

An X-band (9.4 GHz) Bruker E500 spectrometer equipped with a TE102 or a SHQE cavity was used for continuous-wave EPR measurements. For whole cell samples the viability of the cells after the measurement was checked by plating on LB agar plates. Measurements were performed in 2 mm quartz tubes at 100 kHz modulation frequency, 0.15 mT modulation amplitude, 0.6 mW microwave power, 20.48 ms time constant, 81.92 ms conversion time, 1024 points and 15 mT sweep width.

DEER/PELDOR measurements

In-cell PELDOR measurements were performed on a Bruker ELEXSYS E580 EPR spectrometer equipped with a PELDOR unit (E580-400U, Bruker), a continuous-flow helium cryostat (CF935, Oxford Instruments), and a temperature control system (ITC 502, Oxford Instruments). For measurements, 15-20 µL of whole cells containing 15% d_8-glycerol was transferred into 1.6 mm outer diameter quartz EPR tubes (*Suprasil*, Wilmad LabGlass). PELDOR measurements were performed at Q-band frequency (33.7 GHz) using an ELEXSYS SuperQ-FT accessory unit and a Bruker AmpQ 10 W amplifier with a Bruker EN5107D2 dielectric resonator at 50 K. A dead-time free four-pulse sequence with phase-cycled π/2 pulse was used[3] for PELDOR. For the observer pulses, typical pulse length was 32 ns (π/2 and π) and for the pump pulse a 20 ns (π) pulse was used. The observer pulses were set 60 MHz lower than the pump pulse, which was set to the maximum of the echo-detected field swept spectrum. For the outer membrane samples, the measurements were performed on another Elexsys E580 EPR spectrometer fitted with a standalone Q-band bridge with the EN5107D2 resonator. DEER data were acquired with the four-pulse sequence with 16 and 32 ns π/2 and π observation pulses, respectively, and a 32 ns π pump pulse was used. The observation frequency was set 60 MHz lower than the pump frequency, which was positioned to the maximum of the nitroxide spectrum. The deuterium modulations were averaged by increasing the first interpulse delay by 16 ns for 8 steps. The background function arising from intermolecular interactions were removed from the primary data $V(t)/V(0)$ and the resulting form factors $F(t)/F(0)$ were fitted with a model-free Tikhonov regularization to distance distributions with DeerAnalysis2013 software package.[4] The errors in distance distributions arising from uncertainties in background correction was evaluated by systematically varying the background function. *In silico* spin labeling and simulations on the crystal structures were performed using a rotamer library approach with using the MMM 2015[5] software package.

Raw EPR data for the reduction of MTSL by *E. coli* cells

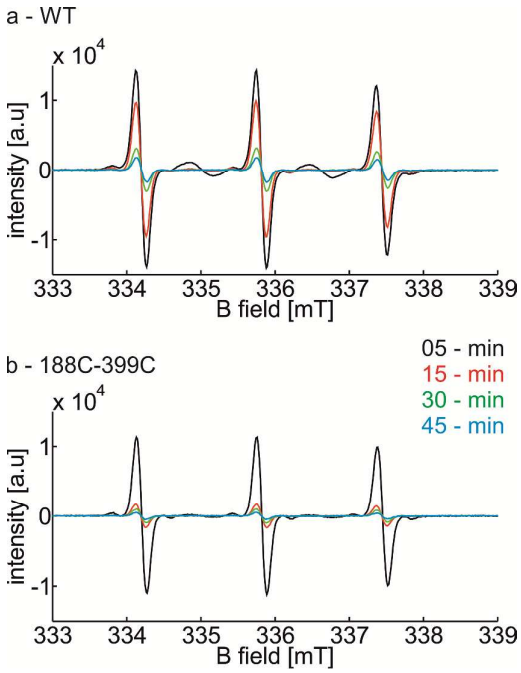

Figure S1. Original RT CW-EPR spectra (analysis shown in Figure 2a) for the reduction of MTSL by *E. coli* cells expressing WT or 188C-399C BtuB obtained at different time intervals after addition of 150 µM MTSL (see material and methods). The broad spectral feature between the hyperfine lines is likely arising from biradical formation.

***In-cell* spin labeling of cysteines located in periplasm or on the plug domain in BtuB**

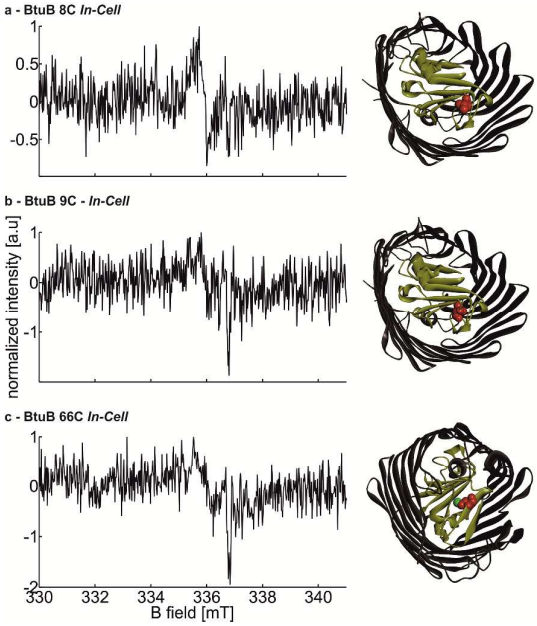

Figure S2. RT CW-EPR spectra for spin-labeling of 8C, 9C (located on the Ton box in periplasm) and 66C (located on the plug/hatch domain) obtained in live *E. coli* cells. Spin-labeling failed for all the three positions. For 8C and 9C, a view of the corresponding residues L8 and V9 as observed from periplasm in the BtuB-Ca^{2+} (1NQG) crystal structure is shown. MMM simulation predicted 73 rotamers for position 8R1 (partition function, 0.35) whereas only one rotamer for position 9R1 (partition function, 0.33). Thus, labeling for 9C might have failed due to limited accessibility as well, whereas 8C could not be labeled most likely due to the reduction of the MTSL in periplasm (Figure 2a). For 66C, a view from top of the corresponding residue I66 in the structure (1NQG) is shown with the terminal carbon atom highlighted in green showing its location into the core of the plug. MMM simulation predicted three roamers (partition function, 0.00) for 66R1 revealing that the site is too tight for labeling.

Simulation of the i*n-cell* RT CW-EPR spectra

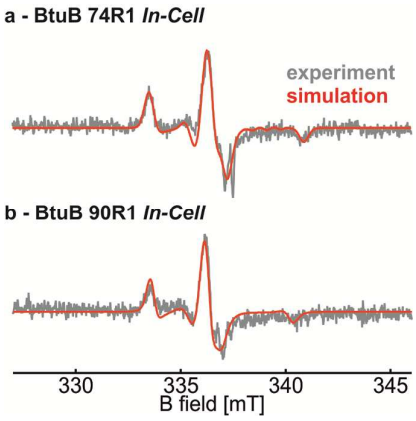

Figure S3. EasySpin[6] simulated RT CW-EPR spectra for 74R1 (a) and 90R1 (b) overlaid on the experimental data obtained in live *E. coli* cells. For 74R1 simulation, g_{xx} = 2.00800, g_{yy} = 2.00600, g_{zz} = 2.00200; A_{xx} = 19.169, A_{yy} = 17.276, A_{zz} = 102.2 (in MHz) and an anisotropic diffusion tensor with an average correlation time of 2.64*10^{-7} s was used. For 90R1 simulation, g_{xx} = 2.00800, g_{yy} = 2.00614, g_{zz} = 2.00186; A_{xx} = 16.217, A_{yy} = 15.426, A_{zz} = 99.800 (in MHz) and an average correlation time of 1.03*10^{-7} s was used.

PELDOR data analysis for whole cell samples

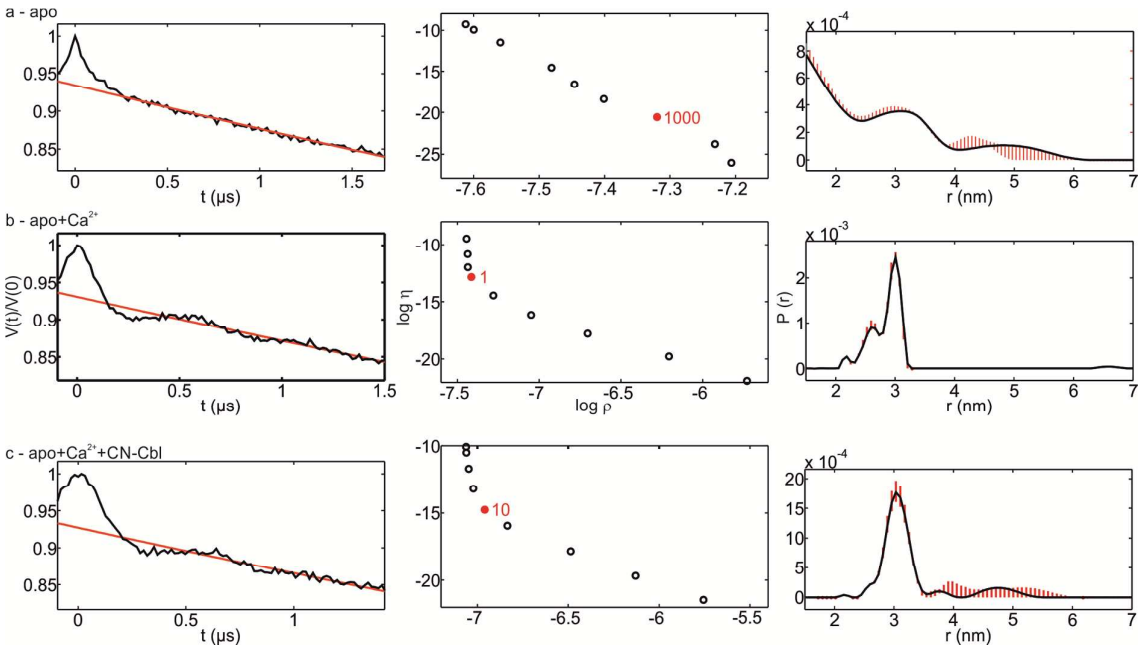

Figure S4. Data analysis was performed with DeerAnalysis[4] software. (a, b, c) Left panels; original PELDOR data for BtuB in different functional states obtained in whole cells (black) with an exponential background function overlaid (red). Middle panels; the corresponding L-curves with the used regularization parameter (α) highlighted in red. The symbol ρ is the mean squared deviation between the experimental and simulated dipolar evolution functions and η is a measure of the roughness of P(r) given by the square norm of the second derivative of P(r) weighted by α. Right panel; error validation for the obtained distance distributions. The beginning of the background function was varied from 200ns to 1000 ns in discrete steps using the validation tool in DeerAnalysis software.

PELDOR data analysis for the (outer membrane) OM samples

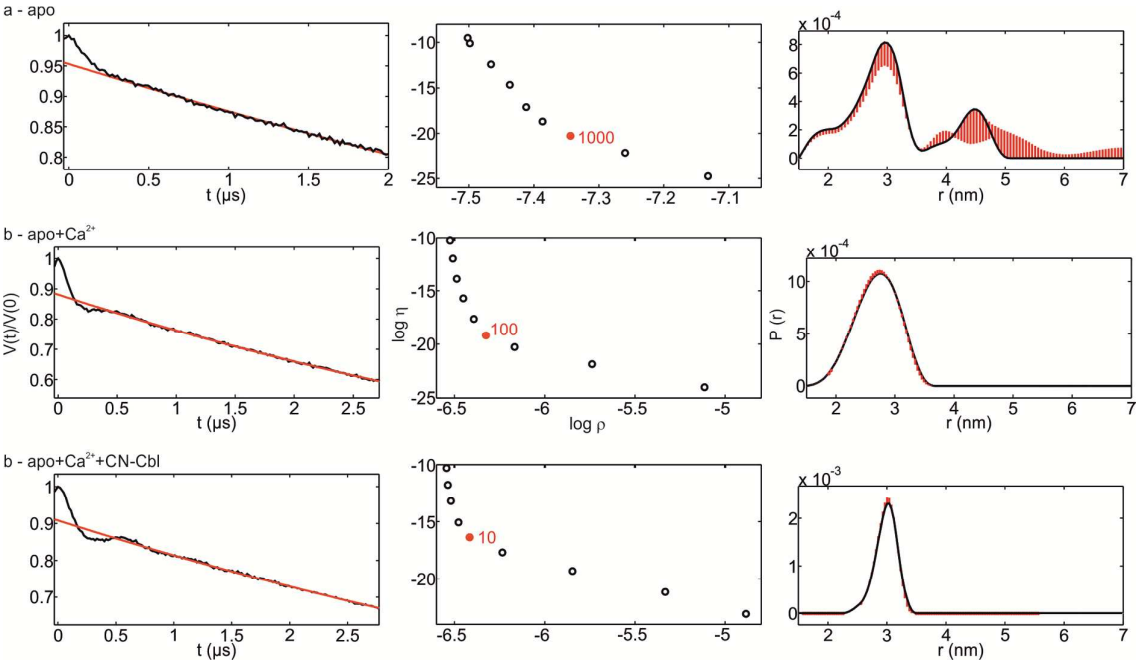

Figure S5. Data analysis was performed with DeerAnalysis software. (a, b, c) Left panels; original PELDOR data for BtuB in different functional states obtained in native outer membranes (black) with an exponential background function overlaid (red). Middle panels; the corresponding L-curves with the used regularization parameter (α) highlighted in red. The symbols ρ and η are explained in Figure S4. Right panels; error validation for the obtained distance distributions. The beginning of the background function was varied from 200 ns to 1500 ns (a) or from 320 ns to 2000 ns for the Ca^{2+} and Ca^{2+}+CN-CBl samples using the validation tool in DeerAnalysis software.

References

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