Table of Contents for Supplementary Material

Material and Methods

Mutagenesis and expression of BtuB

Plasmids harbouring wild-type or 10C, 188C, 404C and 188C-399C mutations in BtuB on a pUC8 backbone were engineered using the Agilent Technologies (Santa Clara, CA) QuikChange Site Directed Mutagenesis Kit. The mutants were overexpressed in the *E. coli* strain RK5016 (*argH*, *btuB*, *metE*).[1] Cells were grown for overnight in minimal media containing 100 μg/mL ampicillin and supplemented with 0.24% w/v glucose, 150 μ*M* thiamine, 3 m*M* MgSO4, 300 μ*M* $CaCl_2$, and 0.01% w/v of Met and Arg.

Isolation of membranes

Following overnight growth in 1 L minimal Media, the cells were pelleted at 6000 rpm for 10 minutes using Sorval SLA-3000 rotor. The cells were suspended in 30 mL of 10 mM HEPES buffer (pH 6.5) and lysed with a French press 2-3 times. The cell debris were removed by centrifugation at 12,000 rpm for 20 minutes in Sorval SS-34 rotor and the supernatant was collected. To isolate the intact membrane or cell envelope (CE), which is composed of outer membrane (OM) plus inner membrane (CM), the supernatant was centrifuged at 100,000 x g for 1.5 hours. To isolate outer membranes alone, the inner membrane was removed by adding 0.5% sarkosyl and the outer membranes were pelleted by centrifugation at 200,000 x g for 1.5 hour. Following centrifugation, the membranes were suspended in 10 mL of 50 mM Tris buffer (pH 7.5) containing 60 mM NaCl.

Spin labeling of E. coli cells and membranes

Following overnight growth, cells from 1 L culture were pelleted and suspended in 50 mL of 50 mM Tris buffer (pH 7.5) containing 60 mM NaCl and 0.5% glucose and spin labelled with 20 μM 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate (MTSL or MTSSL, Toronto Research Chemicals, North York, ON, Canada) for 1 hour at room temperature. This MTSL concentration corresponds to about 10 fold molar excess of BtuB expressed in whole cells. Following incubation, cells were pelleted and washed 2 times and suspended into 2.5 mL total volume with the same buffer and kept on ice. The viability and number of cells in this final suspension was checked by spreading serial dilutions on LB-agar plates. The membrane fraction was suspended in 10 mL of 50 mM Tris buffer (pH 7.5) with 60 mM NaCl and was spin labelled with 40 μM MTSL at room temperature for 1 hour. Following incubation, the membranes were washed until free spin label was completely removed and suspended into 500 μL of the same buffer.

Synthesis of spin labeled cyanocobalamin (TEMPO-CNCbl)

TEMPO-CNCbl was synthesized using a two-step reaction, in which the ribose-5'-hydroxyl was first activated with 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT) and subsequently reacted with 4-amino TEMPO.

Previously, this scheme was used for attaching cyanocobalamin to insulin.[2] The synthesis was performed under argon atmosphere using dry solvents. For the synthesis, 0.1 g of cyanocobalamin was dissolved in 50 mL DMSO and 0.036 g of 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT, Sigma) was added and the reaction mixture was stirred for 30 minutes at room temperature. Following incubation, 0.13 g of 4-amino-TEMPO (Sigma) was added and the reaction mixture was stirred at room temperature for overnight. TEMPO-CNCbl was precipitated with 200 mL of 1:1 mixture of acetone and diethylether. The precipitate was centrifuged at 4^0C for 15 min at 4000 x g. The supernatant was precipitated again and the precipitate was removed by centrifugation as before. The pooled precipitate was washed with acetone, centrifuged, dried overnight under air and afterwards freeze-dried. The yield of the crude product was 0.069 g corresponding to 69 % of the theoretical yield.

Purification and characterization of TEMPO-CNCbl

The crude reaction product was purified by high performance liquid chromatography (HPLC, Agilent 1200 series) on a BDS-C18 column (5 μM, 2x250 mm from Hewlett-Packard) with detection at 254 and 316 nm. An 8 μL volume of a 1 mM stock of the TEMPO-CNCbl was injected and eluted with 1 mL/min flow rate with 400 bar pressure at room temperature. The following solvent system was used for elution: solvent A: water; solvent B: methanol and elution was performed at a linear gradient of 15-60% B in 40 min. MALDI-ToF -MS measurements were performed on a Voyager STR Workstation DE pro (Applied Biosystems) with 100 kW laser (337 nm) peak power using a matrix made of 2,5 Dihydroxybenzoic acid and 6-Aza-thiothymine. The LC-ESI-MS measurements were performed either with a ThermoFisher Surveyor MSQ or Shimadzu LCMS-2020 system. A 50:50 (v/v) mixture of methanol/water (ThermoFisher) or 0.1% acetonitrile/formic acid (Shimadzu) solvent system was used for column elution. Other conditions: capillary voltage - 3-4 kV, dry gas (N_2) pressure - 5 bar, mass range 80-2000 Da, detection: single quad.

Growth assay with TEMPO-CNCbl

E. coli strain RK5016 (*argH*, *btuB*, *metE*) cells,[1] which can grow only in presence of methionine or CNCbl, was grown at 37^0C for overnight on minimal media agar plates containing 0.24% w/v glucose, 150 μ*M* thiamine, 3 mM MgSO4, 300 μM $CaCl_2$, 0.01% w/v of Arg and 1 μM TEMPO-CNCbl (peak (iii) separated from RP-HPLC shown in Fig. S3E). Following incubation, the ability of TEMPO-CNCbl to support growth was confirmed by observing for colony formation on the plates.

Estimation of BtuB and its labeling efficiency in whole cells and outer membranes (OM)

TEMPO-CNCbl was used to estimate BtuB in OM and whole cells after overexpression. When bound to BtuB, TEMPO-CNCbl produces a broad CW EPR spectrum (Fig. 2). Any free TEMPO-CNCbl can be easily

quantified by measuring the intensity of its high-field hyperfine line, which stands out from the baseline without significant overlap with the signals coming from BtuB-bound TEMPO-CNCbl. This method works only if the K_D of this interaction is much lower than the total concentration of BtuB and TEMPO-CNCbl. Cyanocobalamin binds to BtuB with high affinity (K_D < 1 nM)[3] and it is possible that its modification with TEMPO increases K_D. We did not observe any EPR signal corresponding to free (unbound) form when 10 μM TEMPO-CNCbl was added to whole cells or outer membrane preparations, indicating that the K_D for the interaction is lower than 10 μM. Thus we added increasing amounts of TEMPO-CNCbl to whole cells and OM and quantified free TEMPO-CNCbl if any, by comparing the intensity of the high-field hyperfine line with that for an external standard (100 μM 4-amino TEMPO). The amount of BtuB ([BtuB]) was estimated by subtracting the amount of free-TEMPO-CNCbl from the total amount of TEMPO-CNCbl added. For whole cells, 100 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Sigma) was added to eliminate the proton motive force (pmf) and prevent transport of TEMPO-CNCbl into the periplasm. The spin concentration ([spin]) after MTSSL labelling of whole cells or OM was calculated from the double integral of the RT CW EPR spectra using an external standard (100 μM 4-amino TEMPO). The labelling efficiency for whole cells or OM was calculated by multiplying the ratio of [BtuB] (obtained using TEMPO-CNCbl) to [spin] (from the double integral of the MTSSL labelled OM or whole cells) with 100. Here it is assumed that all BtuB present in whole cells or OM are labelled with MTSSL. The modulation amplitudes obtained (~ 8 %, see Fig. 3 and 4) in our PELDOR experiments with OM samples are in agreement with this assumption.

Calculation of rotamer library for TEMPO-CNCbl

A rotamer library for TEMPO-labelled vitamin B_{12} was generated on the basis of a DFT computation of the geometry of 5'-labelled ribose and a Monte Carlo search of torsion angles space. The DFT computation was performed on spin-restricted Kohn-Sham B3LYP/VDZP level with ORCA 2.8.[4] In Monte-Carlo sampling torsion angles of five rotatable bonds starting with the 4'-5' bond of ribose were varied. Torsion and non-bonding interaction potentials were taken from the universal force field (UFF)[5] and all bond lengths and bond angles were kept fixed. The torsion potentials were considered in random selection of test conformations by assuming a Boltzmann distribution at 298 K. An estimate for the minimum non-bonding energy was obtained from a pre-run with 1'000'000 Monte Carlo trials. In the production run, only conformations were accepted with a non-bonding energy that led to at least 1% of the Boltzmann population of the conformation with minimum non-bonding energy that was encountered during the pre-run. The ensemble of 20'000 of such low-energy conformations was reduced to 144 rotamers by hierarchical clustering in torsion-angle space. The UFF non-bonding energy between rotamer atoms on the one hand and vitamin B_{12} atoms not included in the rotamer construct as well as protein atoms on the other hand is considered during attachment of the rotamers with the

MMM software (www.epr.ethz.ch/software/index), omitting the phosphorus atom of vitamin B_{12} that is directly bonded to the construct. Rotamers are attached by superposition of a right-handed coordinate frame defined by C4' (origin), the ring oxygen (on the *x* axis), and C3' (in the xy plane) of ribose. Quality of the geometry resulting from such attachment was tested on PDB structures 1CCW, 1EGM, 1ET4, 1N2Z, 1N4A, 1NQH, 2GSK, 3M8D, and 4KKI. In most cases the remaining atoms common to the construct and vitamin B_{12} superimpose within a few tenths of an Angstrom or better. We note, however, that quality of the geometry of vitamin B_{12} in x-ray crystal structures of proteins may not be as good as backbone and side group geometry of the protein itself, which could lead to a somewhat larger prediction error for distance distributions compared to the case where both labels are attached to cysteine residues.

Continuous-wave EPR measurements

Continuous-wave (CW) EPR measurements were performed at X-band frequency (9.4 GHz) using a Bruker E500 spectrometer equipped with a TE102 cavity. Experimental parameters: 100 kHz modulation frequency, 0.1 mT modulation amplitude, 0.6 mW microwave power, 20.48 ms time constant, 81.92 ms conversion time, 1024 points, 15 mT sweep width. The EPR signal was recorded as the first derivative of the absorption signal. For *E. coli* whole cells, the viability of the cells after the EPR measurement was confirmed by plating on LB agar plates.

PELDOR measurements

For PELDOR measurements, 15-20 μL of whole cells or OM samples with TEMPO-CNCbl at 1:1 molar ratio (when present) containing 15% deuterated glycerol was transferred into 1.6 mm outer diameter quartz EPR tubes (*Suprasil*, Wilmad LabGlass). Pulsed EPR data were recorded on an ELEXSYS E580 EPR spectrometer (Bruker) 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). Experiments were performed at Q-band frequencies (33.7 GHz) using an ELEXSYS SuperQ-FT accessory unit and a Bruker AmpQ 10 W amplifier with a Bruker EN5107D2 cavity at 50 K. For PELDOR experiments, the dead-time free four-pulse sequence with phase-cycled π/2-pulse was used.[6] Typical pulse lengths were 32 ns (π/2 and π) for the observer pulses and 20 ns (π) for the pump pulse. The pump pulse was set to the maximum of the echo-detected field swept spectrum and the observer pulses were set 60 MHz lower. The deuterium modulations were averaged by increasing the first interpulse delay by 16 ns for 8 steps. To obtain distance distributions, the normalized primary PELDOR data $V(t)/V(0)$ were processed to remove the background function from intermolecular interactions and the resulting form factors $F(t)/F(0)$ were fitted with a model-free Tikhonov regularization to distance distributions with DeerAnalysis2013 software package.[7] The MMM 2014

software package was used for *in silico* spin labeling and simulation of distance distributions based on the crystal structures using a rotamer library approach.[8]

Supplemental Table

Supplemental **Table 1:** MTSSL labeling efficiency for BtuB mutants in whole cells and OM.

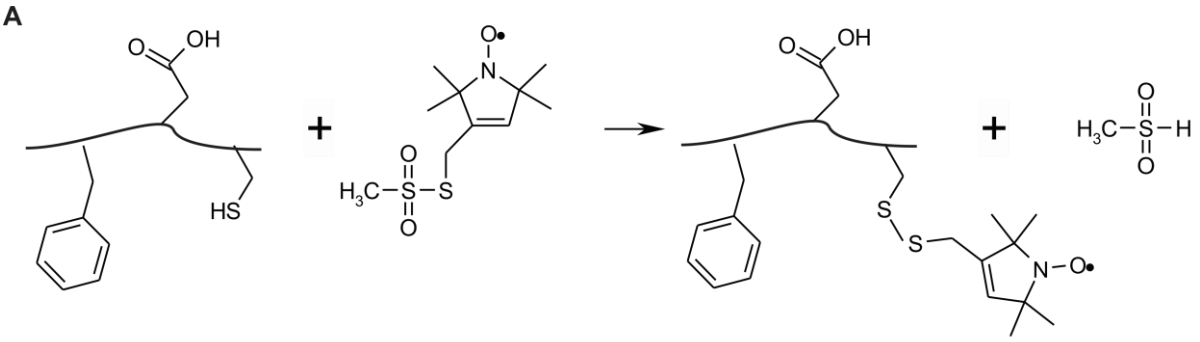
Table 1. Calculated labelling efficiency for BtuB cysteine mutants in intact *E. coli* cells and isolated outer membranes. Cells or OM were prepared from a 1 L overnight culture (see Materials and Methods). Spin concentrations were estimated from the double integral of the room temperature CW EPR spectrum of MTSSL labelled BtuB and the amount of BtuB was quantified using TEMPO-CNCbl, as described in Materials Methods. A 15-20% error is estimated for the given values of BtuB labelling.

Position	[spin, μM] [a]	[BtuB, μM] [b]	% spin labelling[c]
WT whole-cell	below detection	25	Cys-less control
188 whole-cell	30	29	97%
WT - OM	77	21.5	Cys-less control
188 - OM	100	23	23
404 - OM	100	20	20

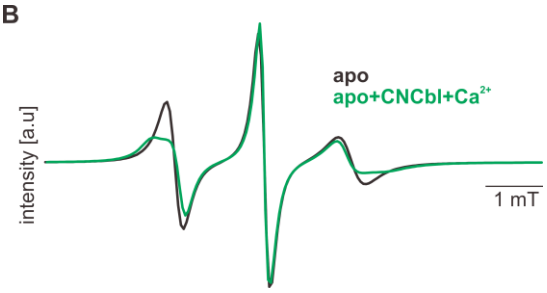
[a] MTSSL labelled BtuB. [b] Obtained using TEMPO-CNCbl binding. [c] Calculated as ([b] / [a]) x 100.

Supplemental Figures

Supplemental **Figure 1:** MTSSL labeling and room-temperature continuous wave (RT CW) EPR spectra for 188R1 in POPC vesicles.

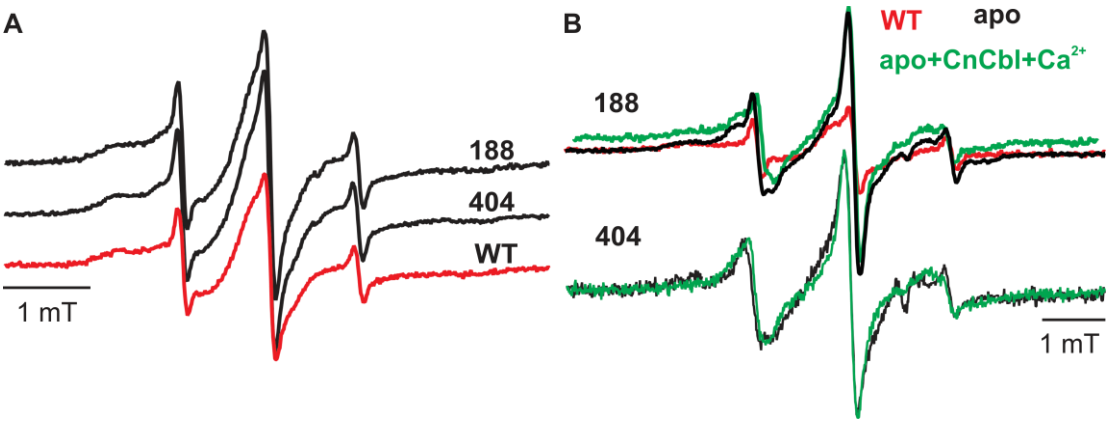


A) Reaction scheme of methanethiosulfonate spin label (MTSSL) with a free cysteine to produce the R1 side chain, adapted from.[9]



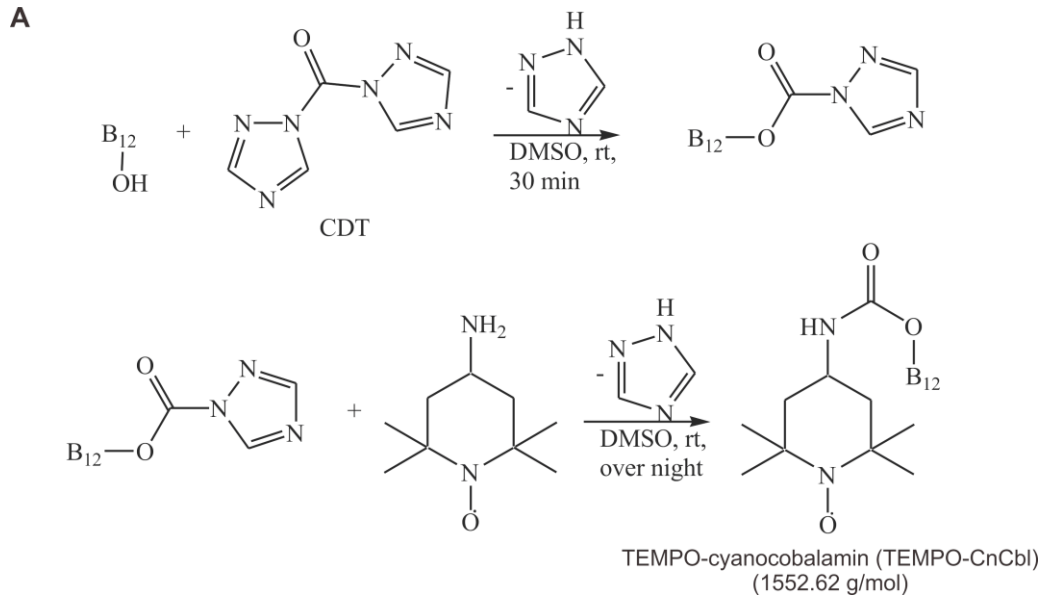
B) 188R1 spectra in apo-state (black line) and in presence of 300 µM vitamin B_{12} (CNCbl) and 1.4 mM Ca^{2+} (green) obtained in POPC vesicles. The data has been published previously.[10]

Supplemental **Figure 2:** Labeling of BtuB in cell envelope (CE, OM+CM) and isolated OM.

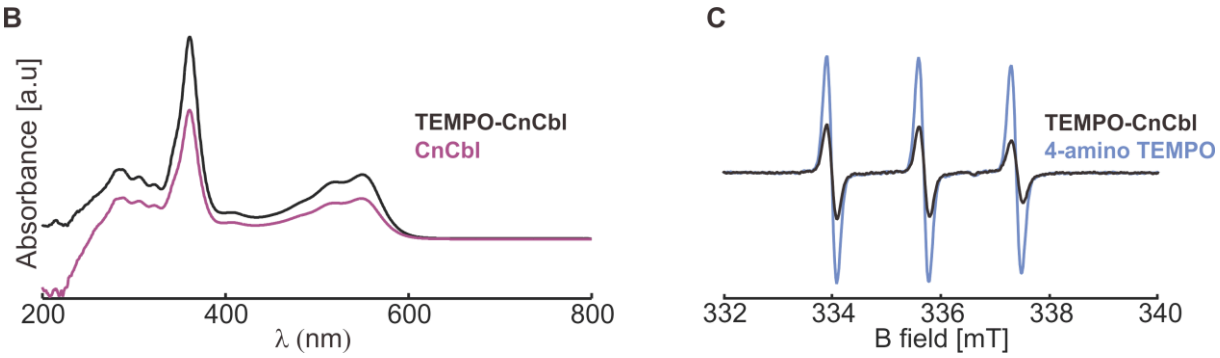


A) X-band RT CW EPR spectra in CE. Membranes were isolated from a 1 L overnight culture of *E. coli* RK5016 cells expressing WT, 188C or 404C BtuB. MTSSL labeling was performed as described in Materials and Methods. The spectra were acquired under identical conditions for a quantitative comparison. Similar shape and intensity of the spectra for WT, 404R1 and 188R1 samples reveal a large non-specific signal. B) RT CW EPR spectra in OM. The CM was selectively removed by sarkosyl solubilization and MTSSL labeling was performed on isolated OM. Even after removal of CM, a significant amount of signal originates from non-specific labeling as evident from the WT control spectrum. Addition of the ligands (100 μM CNCbl + 1 mM $CaCl_2$, the spectra are shifted for clarity) did not induce any visible changes for 188R1 spectra. For 404R1, the presence of these ligands induced changes similar to those observed in whole cells. A one-to-one comparison to the spectra obtained in whole cells is not possible due to a large fraction of signals arising from non-specific labeling.

Supplemental **Figure 3:** Synthesis and characterization of TEMPO-CNCbl.

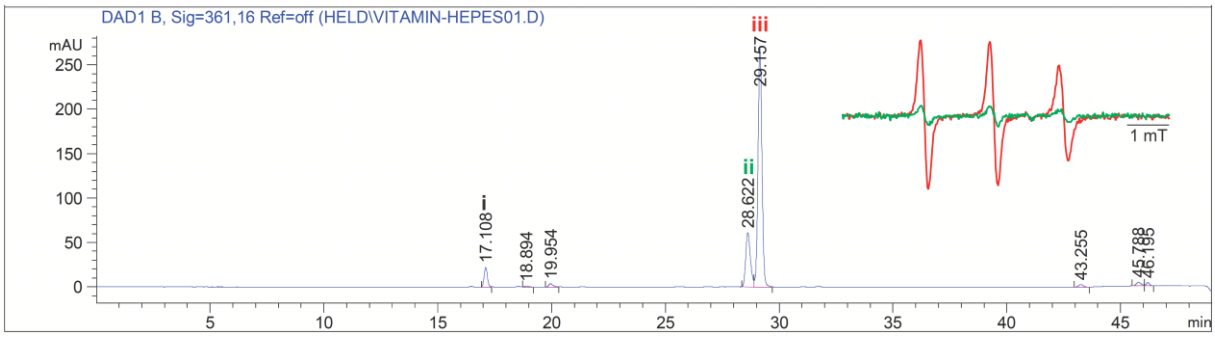


A) Reaction scheme for the attachment of 4-amino TEMPO to cyanocobalaminm (CNCbl, 1355.38 g/mol). CNCbl and CDT were incubated in DMSO at room temperature (rt) for 30 minutes. Following incubation, 4-amino TEMPO was added and incubated for overnight at room temperature and TEMPO-CNCbl (1552.62 g/mol) was purified as described in Materials and Methods.



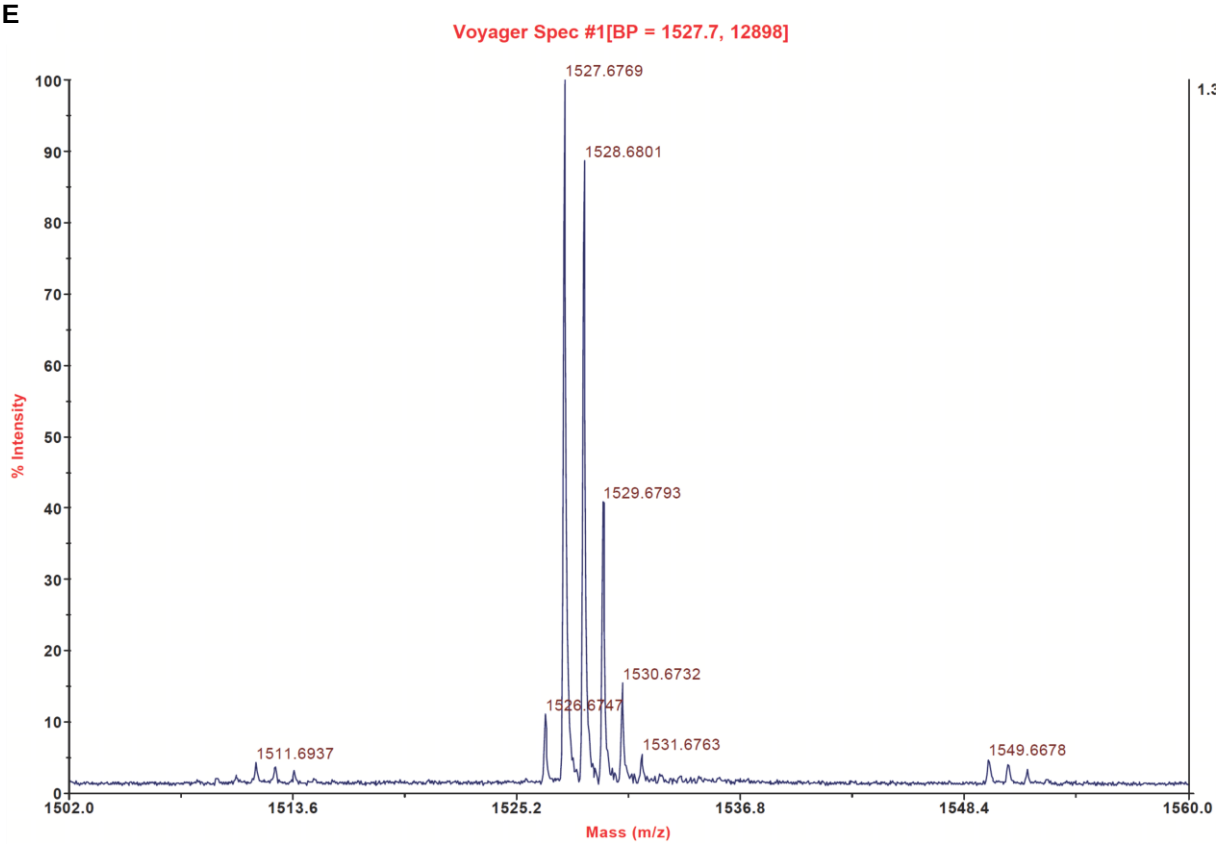
B) The UV-vis absorbance spectra for 50 μM CNCbl and 50 μM TEMPO-CNCbl (after precipitation, calculated with the molecular mass of TEMPO-CNCbl). Modification of CNCbl with TEMPO gives some changes in absorbance particularly at the lower wave lengths. A quantitative analysis by A_{361} (calculated with $\varepsilon = 27108\ cm^{-1}M^{-1}$ for CNCbl) revealed that the crude mixture contained only 31.8 μM cobalamin constituting 63.6% of the total weight. C) RT CW EPR spectra of the above sample compared to a 50 μM 4-amino-TEMPO standard. Quantitative analysis of the spectra (by double integration) reveals that crude reaction mixture contains 27 μM spins constituting 54% of the total weight, which is lower than the cobalamin content determined by UV. This reduction of cobalamin content from EPR determination is mostly due to a fraction of TEMPO-CNCbl existing in reduced from (see the HPLC data below).

D

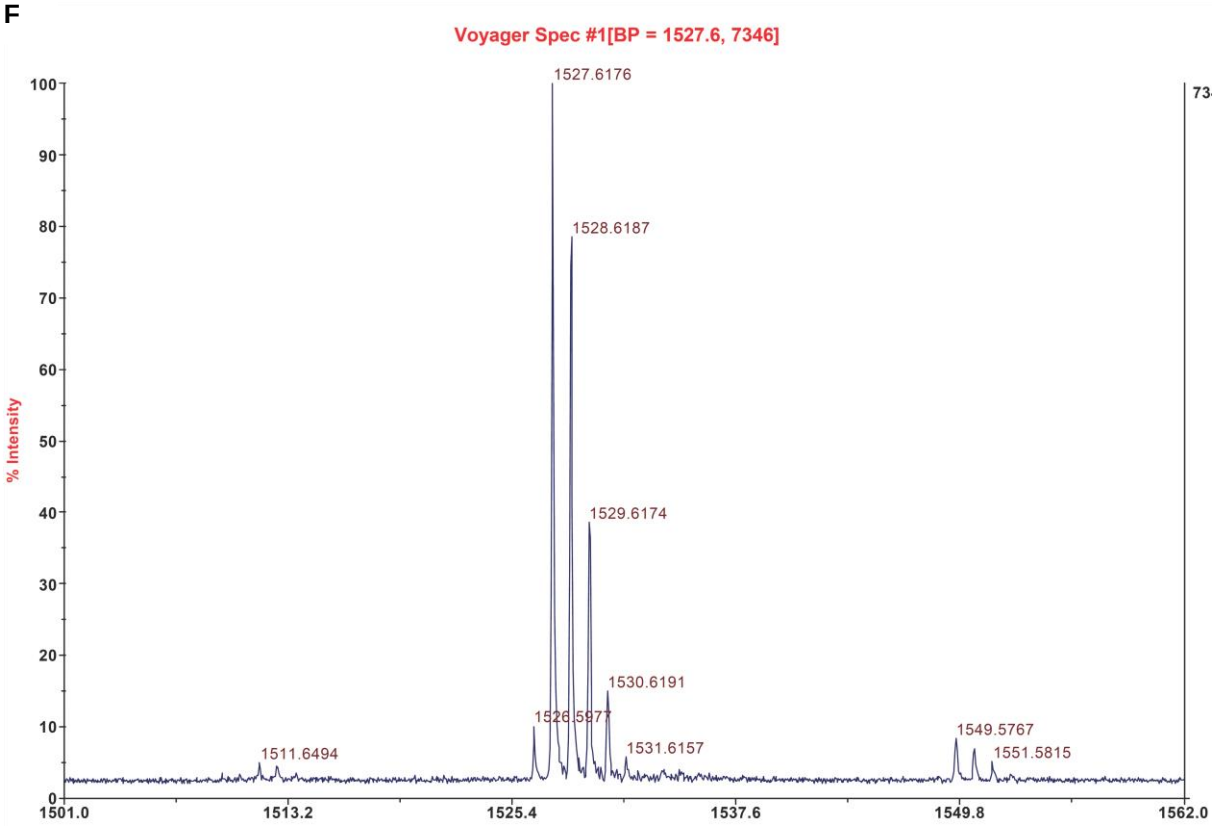


D) RP-HPLC of TEMPO-CNCbl preparation (after precipitation) with detection at 316 nm. Peak at t_R = 17.108 (i) is unreacted CNCbl (1355.38 g/mol) as identified with the standard. Peaks at t_R = 28.622 (ii) and t_R = 29.157 (iii) are TEMPO-CnCbl peaks (1552.62 g/mol). Both peaks gave very similar mass spectra (see panels E-F). RT CW EPR spectra of the peaks (ii) and (iii) are given as inset. Peak (ii) gave a very weak EPR signal, which most likely comes from small overlap with peak (iii) during fraction collection. Thus, peak (ii) contains TEMPO-CNCbl in a reduced form.

E

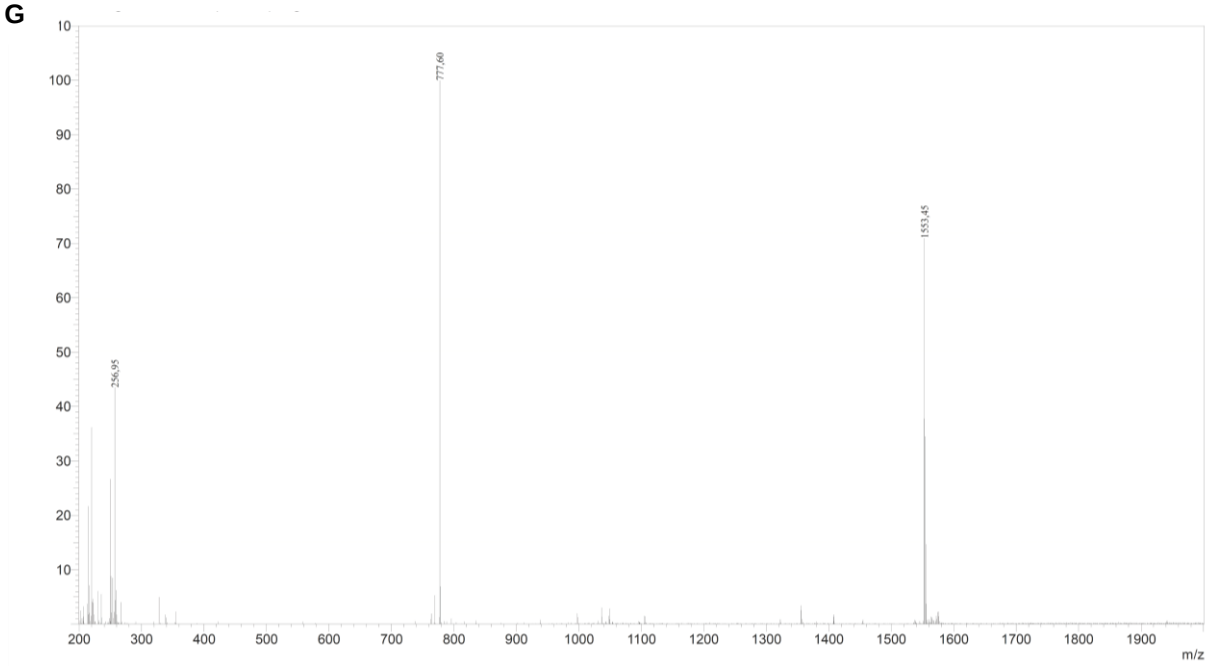


F



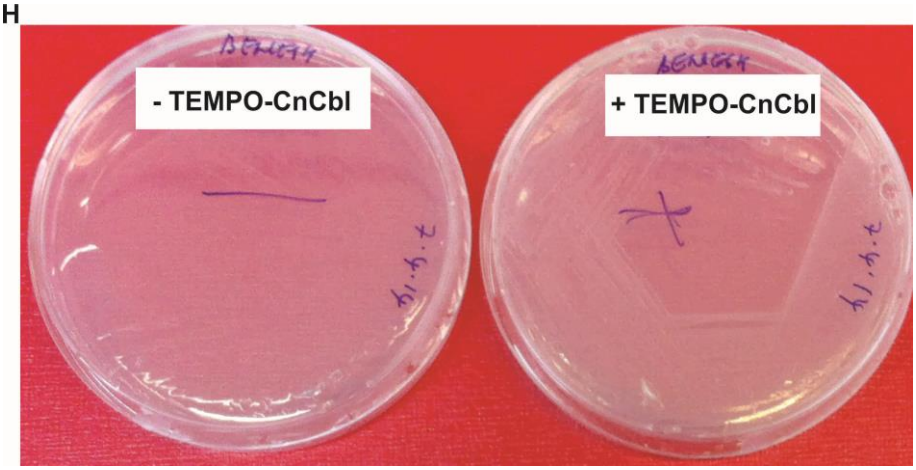
E, F) MALDI-ToF mass spectrum of peak (ii) and peak (iii) respectively separated from the RP-HPLC chromatogram (see panel D). Prominent peaks in the spectra are close to the molecular mass of TEMPO-CNCbl (1552.62 g/mol).

G



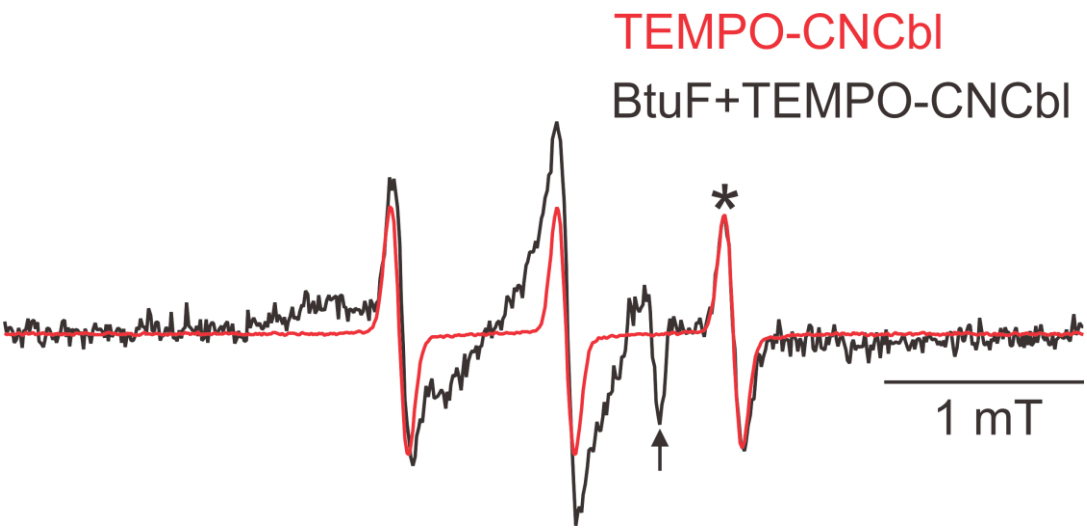
G) LC-MS spectrum of peak (iii) isolated from RP-HPLC (see panel E). The peak at *m/z* 1553.45 is close to the molecular mass of TEMPO-CNCbl (1552.62 g/mol) and the peak at *m/z* 777.6 is close to the half of the molecular mass.

H



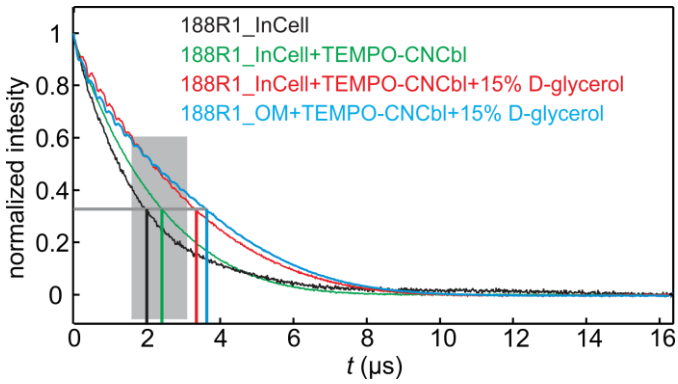
H) Growth assay for TEMPO-CNCbl with *E. coli* cells. *E. coli* strain RK5016 (*argH*, *btuB*, *metE*) cells can grow only in presence of methionine or CNCbl. Cells were grown for overnight on minimal media agar plates containing 0.24% w/v glucose, 150 µ*M* thiamine, 3 m*M* MgSO4, 300 µ*M* $CaCl_2$, 0.01% w/v of Arg and 1 µM TEMPO-CNCbl (peak (iii) separated from RP-HPLC in Fig. S3D). Media containing TEMPO-CNCbl could support the growth of the cells, revealing that modification of CNCbl does not perturb its function.

Supplemental **Figure 4:** Binding of TEMPO-CNCbl with the periplasmic binding protein BtuF.



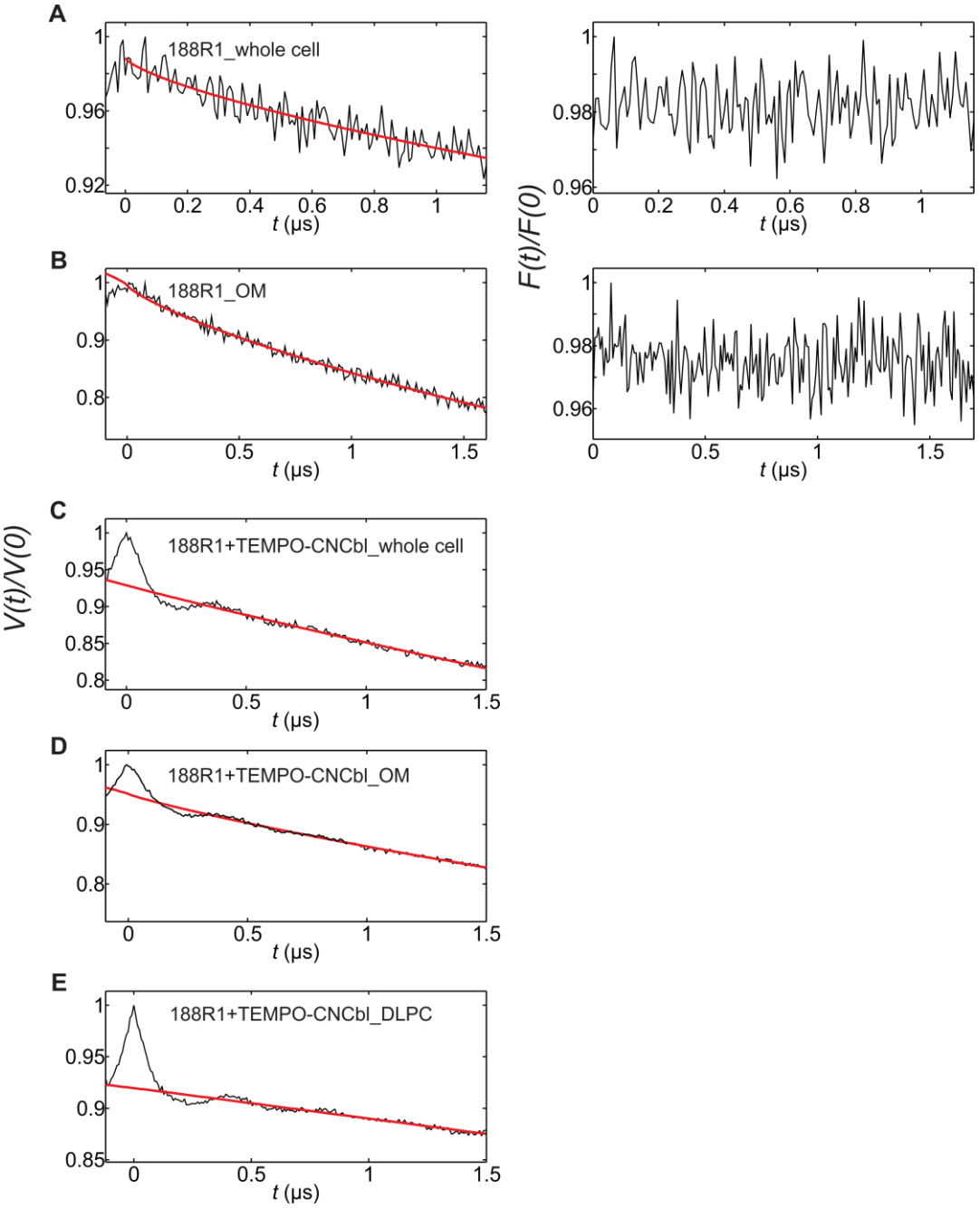
BtuF is the periplasmic cobalamin binding protein, which delivers the substrate to the inner membrane ABC transporter BtuCD-F for transport into cytoplasm. The spectrum was obtained by mixing 40 μM BtuB with 10 μM TEMPO-CNCbl (black). The asterisk indicates the free (unbound) ligand, which is easily distinguished at the high-field hyperfine line. The spectrum of free TEMPO-CNCbl normalized to the high-field hyperfine line is shown for comparison (red). The arrow indicates an artifact from paramagnetic contaminant in the sample tube.

Supplemental **Figure 5:** Spin echo decay in whole cells and outer membranes (OM).



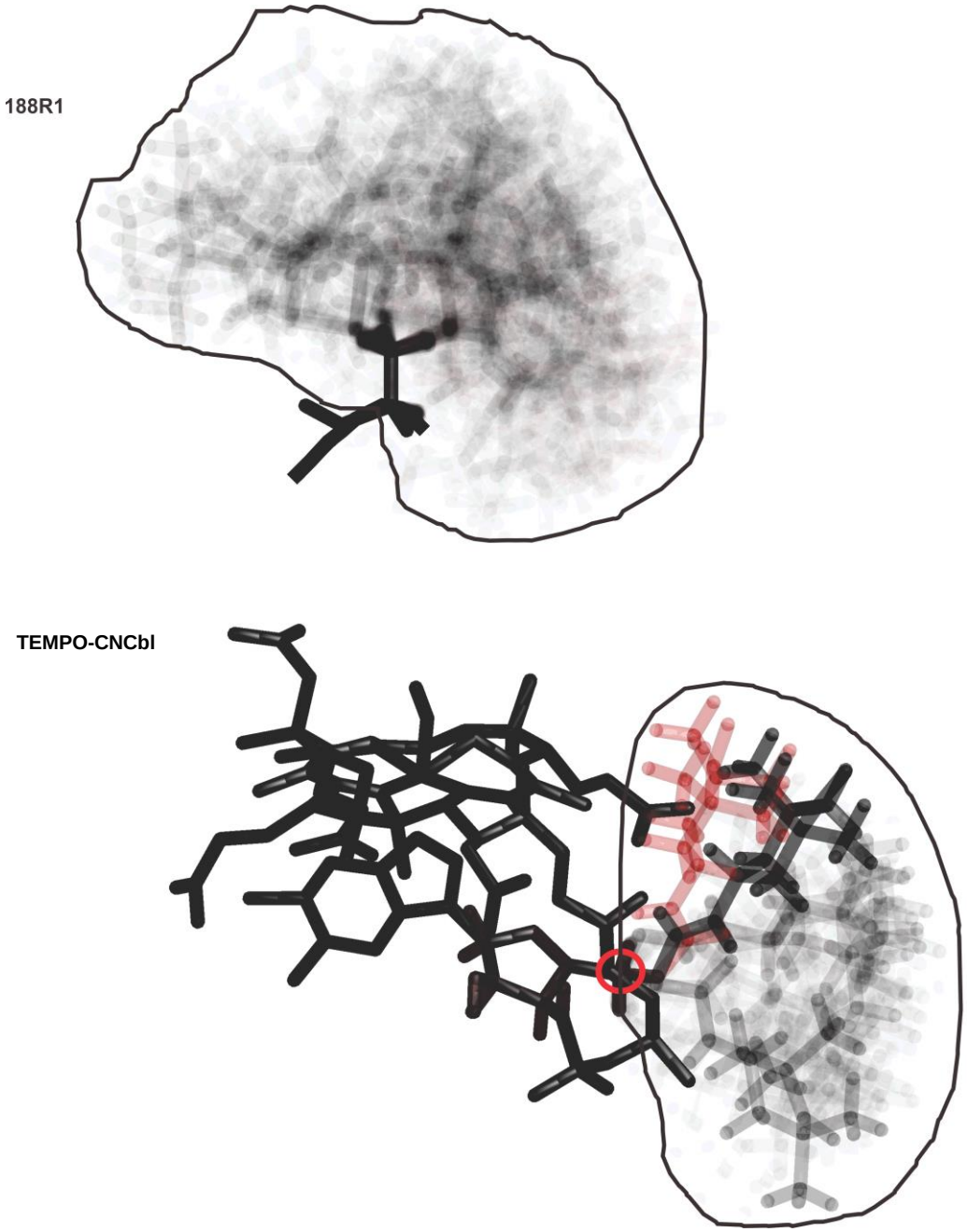
The data were obtained at 50 K by observing at the maximum of the field swept echo at Q-band frequency using a $\pi/2$ - π pulse sequence with 500 ns inter pulse delay, which was increased by 8 ns for 1024 steps. The grey highlighted area indicates the range of τ_2 values used in the PELDOR experiments. The vertical lines indicate the 1/e time, which is approximately equal to the transverse relaxation time (T_2). A reduction of the echo decay rate when TEMPO-CNCbl is added to 188R1 in whole cells (green) is probably due to a slow relaxation of the TEMPO-CNCbl in the binding pocket. Addition of deuterated glycerol (d_8-glycerol) significantly improved the echo decay for both OM and whole cells.

Supplemental **Figure 6:** PELDOR between 188R1 and TEMPO-CNCbl in whole cells and OM.



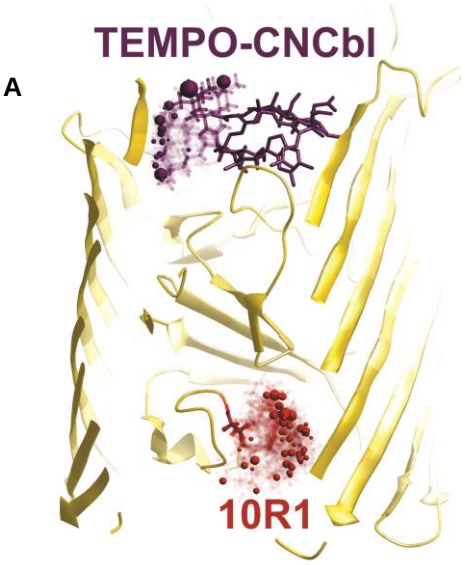
A, B) Normalized primary data *V(t)/V(0)* from PELDOR with 188R1 in whole cells and OM respectively are given on the left with an exponentially decaying intermolecular background (red lines). Corresponding form factors *F(t)/F(0)* obtained after removal of the background with DeerAnalysis2013 software[7] are shown right. C,D,E) Normalized primary data *V(t)/V(0)* from PELDOR between 188R1 and TEMPO-CNCbl in whole cells, outer membranes (OM) and DLPC vesicles respectively with an exponentially decaying intermolecular background (red lines). The corresponding form factors and distance distributions are shown in Fig. 3.

Supplemental **Figure 7:** Simulation of rotamers for 188R1 and TEMPO-CNCbl.

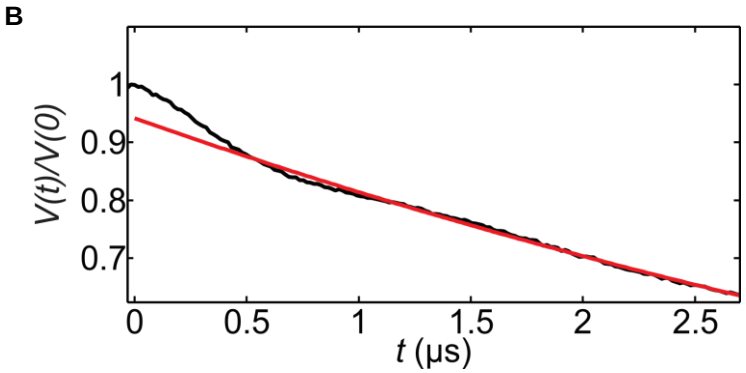


The rotamers were calculated on the BtuB-CNCbl crystal structure (PDB 1NQH) as described in Materials and Methods. For clarity all residues except position 188 and CNCbl carrying the spin labels (R1 side chain and TEMPO respectively) are hidden. The predicted rotamers (115 and 24 rotamers respectively for 188 and CNCbl) lie inside the area marked by black curves. The rotamers contributing to the experimentally detected distances (see Fig. 3) were computed with the "any rotamers" function available in MMM2014 software (http://www.epr.ethz.ch/software/index) and are highlighted in red.[8] For position 188, the identified rotamers are distributed over the entire area (hence lower intensity for the color). For TEMPO-CNCbl, only few of the simulated rotamers contribute to the experimental distance. The red circle inside TEMPO-CNCbl indicate the 5' carbon of the ribose moiety to which TEMPO is attached.

Supplemental **Figure 8:** PELDOR between V10R1 and TEMPO-CNCbl in OM.

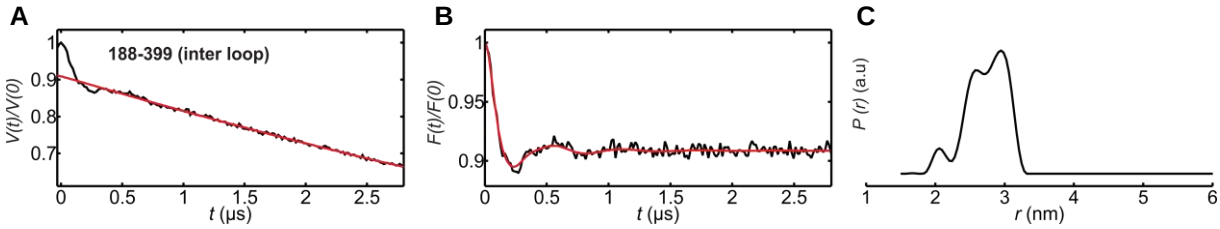


A) Position 10 and TEMPO-CNCbl highlighted with the simulated rotamers on the BtuB-CNCbl crystal structure (PDB 1NQH) using MMM2014 software (http://www.epr.ethz.ch/software/index).[8] The residues of the structure at the front surface was depth cued to visualize the rotamers clearly. The balls indicate the midpoints of N-O bonds and their size corresponds to the probability for each rotamer.



B) Normalized primary data $V(t)/V(0)$ for PELDOR between V10R1 and TEMPO-CNCbl in OM with an exponentially decaying intermolecular background (red lines) fitted with DeerAnalysis2013 software.[7] The corresponding form factor and the distance distribution is shown in Fig. 4.

Supplemental **Figure 9:** PELDOR between 188R1 and 399R1 in OM.



A, B) Normalized Q-band PELDOR trace $V(t)/V(0)$ for PELDOR between 188R1 and 399R1 spin pairs in outer membranes with an exponentially decaying intermolecular background (red lines). B) Corresponding form factor $F(t)/F(0)$ obtained after removal of the background. C) Distance distribution obtained using Tikhonov regularization with DeerAnalysis2013 software.[7]

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