EPR Spectroscopy

Distance Measurement on an Endogenous Membrane Transporter in *E. coli* Cells and Native Membranes Using EPR Spectroscopy**

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Abstract: Membrane proteins may be influenced by the environment, and they may be unstable in detergents or fail to crystallize. As a result, approaches to characterize structures in a native environment are highly desirable. Here, we report a novel general strategy for precise distance measurements on outer membrane proteins in whole Escherichia coli cells and isolated outer membranes. The cobalamin transporter BtuB was overexpressed and spin-labeled in whole cells and outer membranes and interspin distances were measured to a spin-labeled cobalamin using pulse EPR spectroscopy. A comparative analysis of the data reveals a similar interspin distance between whole cells, outer membranes, and synthetic vesicles. This approach provides an elegant way to study conformational changes or protein–protein/ligand interactions at surface-exposed sites of membrane protein complexes in whole cells and native membranes, and provides a method to validate outer membrane protein structures in their native environment.

Membrane protein function often depends upon local and global motions that occur over a wide range of time scales (from picoseconds to milliseconds). These motions may be modulated by the surrounding environment and tools to study membrane proteins under native conditions are of great value.[1] However, determining membrane protein structure

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or dynamics with high resolution in whole cells is challenging and yet to be demonstrated. Like NMR spectroscopy, pulsed electron–electron double resonance (PELDOR), also known as double electron–electron resonance (DEER) is a tool with the potential to examine conformational changes in biomolecules in the cellular environment.[2] PELDOR enables distance measurements in the 1.5–8 nm range between two paramagnetic centers with high precision and reliability.[3,4] It is more sensitive than NMR spectroscopy and there is no size limit to the protein of interest, both important features for in-cell spectroscopy.

Most biomolecules are not paramagnetic, and for EPR spectroscopy they must be modified with an appropriate spin label. Generally, labels are attached by covalently linking a methanethiosulfonate spin label (MTSSL) to cysteines generated by site-directed mutagenesis.[5] Alternatively, spin probes may be incorporated using genetic encoding in response to a nonsense codon.[6,7] Both whole cell labeling of endogenously expressed proteins using MTSSL and genetic encoding have been demonstrated in *E. coli*.[7,8] Distance measurements using PELDOR have also been performed by microinjection of spin-labeled RNA, DNA, peptides, and ubiquitin into eukaryotic cells.[9–13] However, the application of PELDOR on an endogenously expressed biomolecule (RNA, DNA or protein) has not been performed, primarily due to difficulties in obtaining high expression levels and specific and efficient labeling in a complex environment.

Here we introduce a general strategy to make accurate distance measurements on an arbitrary outer membrane protein in intact *E. coli* using PELDOR. Gram-negative bacteria are surrounded by a cell envelop (CE) composed of an outer membrane (OM) and an inner cytoplasmic membrane (CM) separated by a periplasmic space containing a thin peptidoglycan layer. The OM is intrinsically asymmetric and is composed of phospholipids (PL), lipopolysaccharides (LPS), and numerous β-barrel proteins that function in transport, signaling, motility, resistance to toxic compounds, and membrane biogenesis.[14] The function of many of these proteins requires the presence of a proton motive force (pmf) and an interaction with proteins in the periplasm or the CM. For cysteine labeling with MTSSL, the target protein should lack reactive cysteines or have all the natural reactive cysteines replaced. Proteins in the OM of bacteria are generally free of reactive cysteines, thereby minimizing potential sources of non-specific signals.[8,15]

Here, we chose the high-affinity $(K_D < 1\,\text{nM})$[16] vitamin B_{12} (or cyanocobalamin, CNCbl) outer membrane transporter BtuB from *E. coli* as a model system to determine whether in-cell PELDOR can be performed on a membrane

protein. BtuB belongs to a family of proteins termed TonB-dependent transporters (TBDTs) that require the inner membrane ExbB-ExbD-TonB complex and a pmf for function. A conserved segment near the N-terminus of BtuB termed the Ton box acts as an energy-coupling segment and is believed to interact with the C-terminal region of TonB to release substrate into the periplasm.[17] BtuB has been crystalized in apo-, vitamin B$_{12}$-bound[18] and TonB-bound[19] states. In these crystal structures the barrel is occluded with a central hatch (plug) domain and the mechanism by which substrate is released is presently not known.

Typically, 200–300 copies of BtuB are expressed per *E. coli* cell when CNCbl is omitted from the medium,[20] which is far below the level required for a PELDOR experiment (10–50 μM). Therefore we overexpressed wild-type (WT) BtuB and the cysteine mutants 188C and 404C located on extracellular loops 2 and 7, respectively (Figure 1 A), and spin-labeled the *E. coli* whole cells (termed whole cells) with MTSSL. Reaction of the MTSSL with cysteine forms the spin-labeled side chain R1 (Figure S1A), to produce 188R1 and 404R1 derivatives of BtuB.
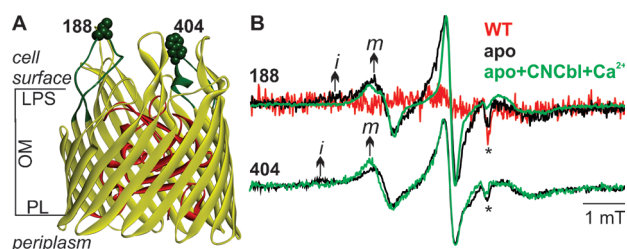

Figure 1. MTSSL labeling of BtuB in live *E. coli* cells. A) Spin-labeled positions (shown in space-filling model) and the loops carrying them highlighted in green using PDB 1NQH. The core domain inside the barrel is shown in red. B) X-band RT CW EPR spectra for MTSSL-labeled *E. coli* expressing WT, 188C or 404C BtuB using a 20 μL suspension containing 2×10⁹ cells (see the Supporting Information). For 188R1, the WT control and the apo-samples were measured under identical conditions for a quantitative comparison. The spectra of 188 apo + CNCbl + Ca²⁺ (100 μM CNCbl + 1 mM CaCl$_2$) is scaled to the intensity of 188-apo sample. For position 404, the spectra are scaled to the same intensity. The arrow indicates the mobile (*m*) and the immobile (*i*) components in the spectra and the asterisk indicates artifacts from sample tubes.

The X-band (9.4 GHz) room-temperature continuous-wave (RT CW) EPR spectrum of whole cells is shown in Figure 1 B and yielded a two-component spectrum composed of a mobile and an immobile component (marked "*m*" and "*i*") for both 188R1 and 404R1. These two components are resolved because of their different correlation times and suggests the existence of two distinct modes of R1 motion at these positions in whole cells. When CNCbl and Ca²⁺ were added to *E. coli*, the immobile components vanish, and for 188R1 the spectra narrowed as a result of stronger averaging of the magnetic anisotropy of R1 reflecting an increased motion of the loop. This observation confirms that over-expression does not affect the ability of BtuB to bind the ligand and undergo conformational changes. An earlier study with 188R1 reconstituted in POPC vesicles revealed a differ-

ent lineshape and the presence of the ligands reduced loop mobility,[21] showing that the dynamics and conformational changes are modulated by the surrounding environment. Unlike the short stability of nitroxide spin labels against reduction observed inside the cells,[7,9,22] the signal intensity for both 188R1 and 404R1 was stable for at least 7 h when the cells were kept on ice. When WT BtuB was expressed at comparable levels to the mutants (see Table S1 in the Supporting Information), the EPR signal was very weak indicating that most of the signal from the cysteine mutants originated from specific reaction with the MTSSL. In contrast to residues 188 and 404 that face the cell surface, residue 10 in the Ton box that faces the periplasm could not be labeled in whole cells. Thus in whole cells, labeling is limited to those surface-exposed residues with direct access to MTSSL from outside.

In addition to whole cells we also isolated CE membranes (OM + CM) following overexpression of the protein. Our attempts to spin label BtuB in these CE membranes was not successful due to a high level of non-specific labeling. Positions 188 and 404 were tested and found to have identical spectra to the WT control samples (Figure S2A). Previously, it was shown that sites in the Ton box of BtuB (residues 6–12) could be labeled with the MTSSL in isolated OM preparations.[15] Therefore, we prepared isolated OM membranes by selective removal of the CM with sarkosyl solubilization.[15,23] As expected, removal of CM reduced non-specific labeling (Figure S2B). However, the spectra for 188R1 and 404R1 still contained significant contributions from non-specific signals, making a direct comparison to the in-cell or POPC spectra difficult. We succeeded in labeling position 399 on loop7 (see Figure S9) and position 10 in the Ton box at the periplasmic face (see Figure 4) for which labeling failed in whole cells.

Non-specific spin-labeling influences PELDOR distance distributions if the resulting dipolar interactions are within the measureable range. If the non-specifically attached labels are far apart or the two cysteines on BtuB are only partially spin-labeled, the modulation amplitude and the signal-to-noise ratio (S/N) are reduced. Another complication could arise from overexpression if this promotes protein oligomerization or aggregation. In order to determine the feasibility of measuring PELDOR signals in native systems with intrinsic background signals, we synthesized a TEMPO-labeled cyanocobalamin (TEMPO-CNCbl, see the Supporting Information; Figure 3 and Figure S3A–H), which should yield a specific distance to another spin label on BtuB. TEMPO-CNCbl binds to both BtuB (Figure 2) and the periplasmic binding protein BtuF (Figure S4). Moreover, TEMPO-CNCbl supported the growth of *E. coli* RK5016 cells on minimal media (Figure S3H), indicating that the modification did not significantly disturb structure and function. Using the binding of TEMPO-CNCbl, we estimated the amount of BtuB in whole cells and OM (see the Supporting Information). For whole cells, we could achieve up to 30 μM BtuB concentration with 97% specific labeling, whereas for the OM preparations we obtain only 20–25% specific labeling because of a large background signal (Table S1).

In a second step, we performed PELDOR measurements using 188R1 in whole cells, OM, and reconstituted DLPC
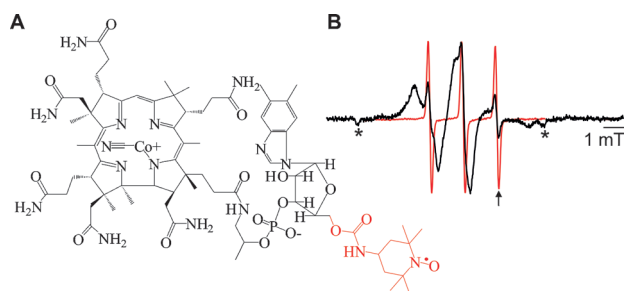
Figure 2. TEMPO-CNCbl structure and binding. A) Structure of TEMPO-CNCbl with the TEMPO and the bonds to the 5′ carbon of ribose highlighted in red. B) RT CW EPR spectra of 10 μM TEMPO-CNCbl (red) or 10 μM TEMPO-CNCbl + 1 mM CaCl₂ + BtuB reconstituted in POPC vesicles (black). The arrow indicates the characteristic resonance position for the third hyperfine line of free TEMPO-CNCbl because of its smaller correlation time and the asterisks indicate artifacts from sample tubes.
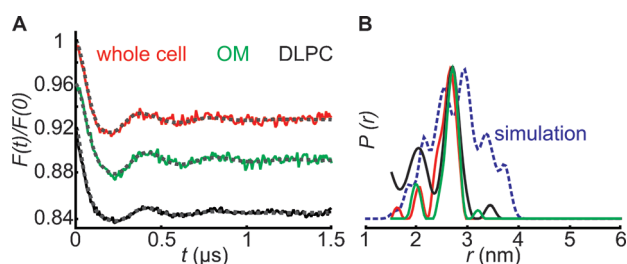

Figure 3. PELDOR between BtuB188R1 and TEMPO-CNCbl spin pairs in whole cells, OM, and DLPC vesicles. The whole cells or outer membrane preparations contained 25–30 μM BtuB after MTSSL labeling, and PELDOR was performed after adding an equal amount of TEMPO-CNCbl. A) Normalized form factors obtained after intermolecular background correction of the primary data (Figure S6 C–E) using a 3D spin distribution with the fittings overlaid in dotted gray lines. B) Distance distributions calculated with the DeerAnalysis2013[25] software with the Tikhonov regularization parameter α set to 10. In DLPC, the modulation amplitude obtained is not directly comparable to the other samples due to the bidirectional orientation and unknown concentration of BtuB. Even with a large fraction of non-specific labeling (Table S1) the OM sample gave about 8% modulation amplitude. Although devoid of non-specific signals, whole cell samples as well gave similar modulation amplitudes, which might be due to the transport of a fraction of TEMPO-CNCbl into the periplasm. The blue line shows the MMM simulation[24] (www.epr.ethz.ch/software/index) for PELDOR between 188R1 and TEMPO-CNCbl on PDB 1NQH.
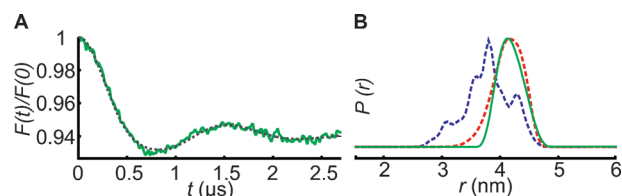

Figure 4. PELDOR between BtuB10R1 and TEMPO-CNCbl spin pairs in OM. A) Normalized form factor obtained after intermolecular background correction of the primary data (Figure S8B) using a 3D spin distribution with the fitting overlaid in dotted gray line. B) Distance distributions calculated using the DeerAnalysis2013[25] software with the Tikhonov regularization parameter α set to 10. The blue dotted line is the distance distribution obtained from the simulation for PELDOR between 10R1 and TEMPO-CNCbl using BtuB-CNCbl structure (PDB 1NQH). The red dotted line is an additional simulation using the "any rotamers" function in MMM software, in which the side-chain packing is disregarded to match the experimental data.

vesicles after addition of TEMPO-CNCbl (in 1:1 molar ratio to BtuB). The presence of 15% [D₈]glycerol significantly improved the spin echo decay both for whole cells and OM samples (Figure S5). In the absence of TEMPO-CNCbl, both whole cells and OM gave only an exponentially decaying signal (Figure S6A,B), demonstrating that overexpression of BtuB does not cause oligomerization or aggregation and that the non-specific labeling particularly in the OM results in spins spatially separated by distances longer than 7 nm. In presence of TEMPO-CNCbl, whole cells gave a PELDOR trace yielding a bimodal distance distribution with mean values at 2.02 ± 0.07 and 2.60 ± 0.15 nm, respectively (Figure 3). Despite the dramatic differences in the surround-

ing environments, the measured distance distributions in whole cells, OM and DLPC vesicles were similar. Remarkably, even with a large amount of non-modulated signals from nonspecific labeling, OM preparations also gave clean PELDOR traces, confirming that there is specific binding of TEMPO-CNCbl to BtuB. To compare the experimental data with the crystal structure, we computed 188R1—TEMPO-CNCbl distance distributions in BtuB-CNCbl structure (PDB 1NQH) using rotamer libraries calculated for R1[24] and TEMPO-CNCbl (see the Supporting Information). In addition to distances seen experimentally, simulations revealed the presence of longer distances (Figure 3), which arose primarily from rotamers of TEMPO-CNCbl seen in the crystal-structure-based simulation that were not populated in the membrane environment (Figure S7).

To determine whether distances could be measured to sites that were not labeled in whole cells, we attempted PELDOR across the OM between 10R1 located in the Ton box and TEMPO-CNCbl (Figure S8A). Interestingly, PELDOR gave a single distance with a mean value at 4.17 ± 0.21 nm (Figure 4). Simulations on the BtuB-CNCbl structure (1NQH) revealed a distance distribution between 2.6–4.75 nm with mean distance at 3.78 ± 0.40 nm (Figure 4). This discrepancy with experimental data may be accounted for by the differences in side-chain packing predicted in the crystal structure when compared to the OM (red line in Figure 4). Additionally, PELDOR between spin labels attached to loop2 and loop7 (Figure S9) in OM indicates that this approach can be used to follow conformational changes of the loops and other flexible regions in native environments.

In summary, we have demonstrated for the first time the application of PELDOR in whole cell and native membrane environments to extract precise distance constraints for an endogenously expressed membrane protein. In this study, the concentration of BtuB used (30 μM) corresponds to about 10^5 copies per cell, which is comparable to the expression level of some endogenous outer membrane proteins. A comparison of 188R1 and TEMPO-CNCbl PELDOR data between three different membrane environments indicates that the lipid composition does not significantly influence the loop conformation. Further measurements with other positions on

BtuB will tell us whether this observation holds in general. OM preparations provide a versatile tool to study membrane proteins and unlike the alternate approaches such as nanodiscs,[26] it does not require the tedious processes of solubilization, purification, and membrane reconstitution of the target protein. The approach presented here provides an opportunity to validate outer membrane protein structures and to make new structural and functional investigations of several outer membrane macromolecular complexes critical for gram-negative bacterial physiology under native conditions.

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