EPR spectroscopy

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PELDOR Spectroscopy Distance Fingerprinting of the Octameric Outer-Membrane Protein Wza from *Escherichia coli*.**

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Membrane proteins account for over one-fifth of the encoded proteins, but their crystallization is challenging, particularly for multiprotein complexes such as the polysaccharide export system of *Escherichia coli*.[1,2] One major component of this system is the translocation channel Wza, an octameric outer-membrane protein of 320 kDa whose closed-state crystal structure has been recently determined.[3] Wza is thought to interact with different other proteins which, in part, reside in the inner membrane and cytosol, to form a periplasm-spanning molecular machine.[1,2] The open-state structure of Wza is presumably stabilized during interaction with other proteins, and X-ray crystallography alone may not give a view of this dynamic complex. Pulsed electron–electron double resonance spectroscopy (PELDOR) is a powerful tool for measuring distances up to 80 Å.[4–6] Recently, the approach has been applied to study the maltose ATP binding cassette membrane protein complex and to quantify the internal motions during the catalytic cycle.[7] However, the approach has yet to be applied to large, highly symmetric integral membrane proteins such as Wza. This is an important gap, as many cellular processes involve highly symmetric membrane proteins. We show herein that PELDOR spectroscopy is well-suited for the study of such a system, and a comparison of the PELDOR data with the crystal structure demonstrates the accuracy of the distance fingerprint. This fingerprint provides a convenient ruler by which to assess conformational changes.

Two spin-labeled Wza species were made by expressing the single mutants G58C and Q335C of Wza and then reacting these mutants with the nitroxide MTSSL (methanethiosulfonate). Mass spectrometry was used to confirm the labeling. Since Wza is an eightfold-symmetric multimer, the eight labels (one in each monomer) give rise to four principle distances (Figure 1).

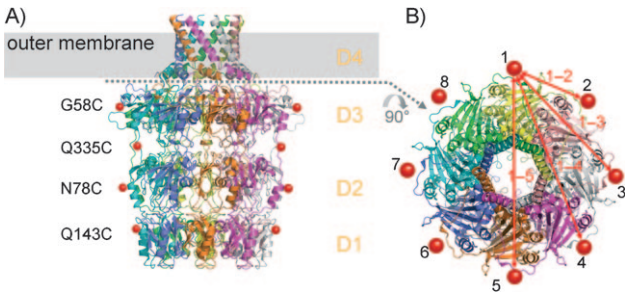

Figure 1. Structure of Wza. A) Each Wza monomer is colored differently to indicate the octameric structure. Domains D1–D4 are labeled, and the position of the outer membrane is indicated by the gray bar. Red spheres indicate where nitroxide spin labels were attached. B) View from the extracellular space; the colors correspond to (A).

PELDOR experiments were performed on these proteins solubilized in *n*-dodecyl-β-D-maltopyranoside (DDM) micelles, yielding modulated time traces and distance distributions (Figure 2 A,B). Two distinct distance peaks could be measured for each mutant, for Wza_{Q335C} at 28.6 and 51 Å and for Wza_{G58C} at 36.7 and 66 Å (Table 1). Taking the additional length of each label into account (ca. 9 Å), these distances agree well with the C_β–C_β distances 1–2 and 1–3 (Figure 1 B) inferred from the crystal structure (Table 1).

The purification of Wza is time-consuming, so we sought to investigate whether a soluble version of the protein could be produced that retained the same structural properties as full-length Wza. We expressed a truncated Wza 24–345 mutant (sWza; see the Supporting Information), which lacks the signal sequence and the C-terminal transmembrane domain D4. The structure of sWza was essentially identical to the corresponding domains from the full-length protein (see the Supporting Information). As the C-terminal transmembrane helices are unlikely to be involved in protein–protein complex formation, sWza is a suitable system for further study. A set of four mutant constructs was made ($sWza_{Q335C}$, $sWza_{G58C}$, $sWza_{N78C}$, and $sWza_{Q143C}$) spanning the full length and width of octameric sWza. PELDOR experiments on $sWza_{Q335C}$ and $sWza_{G58C}$ give the same peaks identified for the corresponding full-length construct, but the spectra appear sharper. PELDOR measurements on $sWza_{N78C}$ and $sWza_{Q143C}$ yielded principle distances 1–2 and 1–

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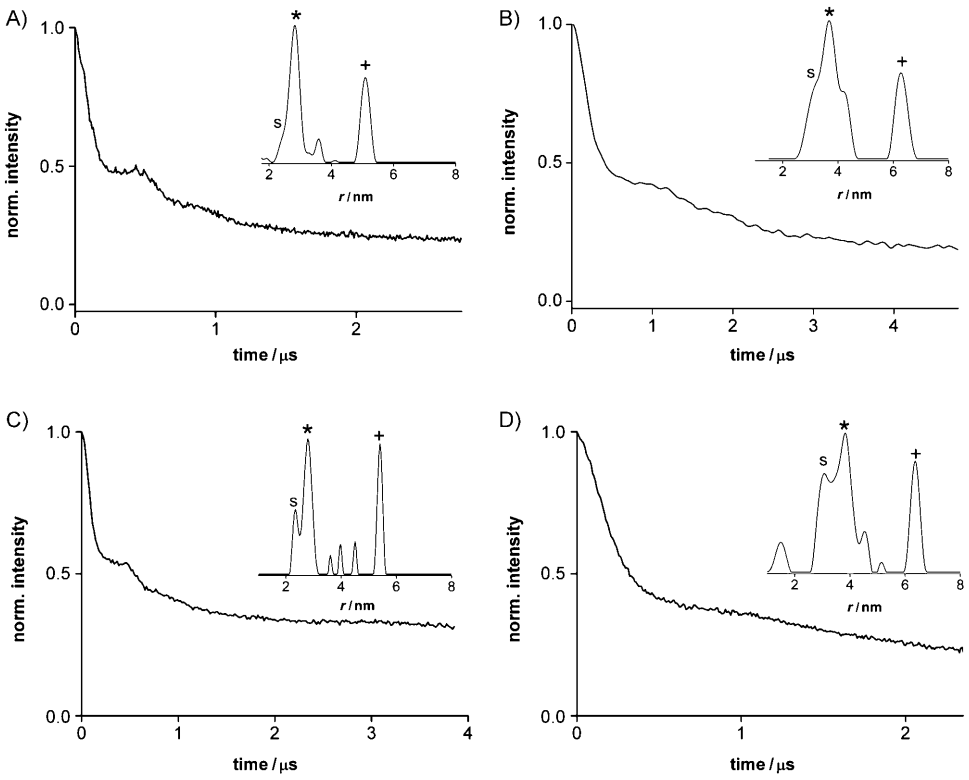

Figure 2. PELDOR time traces and distance distributions (insets) for A) Wza_{Q335C}, B) Wza_{G58C}, C) $sWza_{Q335C}$, and D) $sWza_{G58C}$. The labels (*) and (s) indicate the two conformers for the principle distance 1–2 and the label (+) marks principle distance 1–3.

Table 1: Distances obtained for Wza and sWza by PELDOR spectroscopy and X-ray crystallography.

Label		Spin–Spin Distances [Å]					
		1–2			1–3		
		PELDOR[a]	C_β–C_β[b]	X-ray[c]	PELDOR[a]	C_β–C_β[b]	X-ray[c]
Wza	Q335C	23.6, 28.6	24.9	–	51	45.9	–
	G58C	31.8, 36.7	33.5[b]	–	66	62.3[b]	–
sWza	Q335C	23.7, 28.1	24.9	23.4, 29.0	54	45.9	53.1
	G58C	32.1, 36.6	33.5[d]	–	64	62.3[b]	–
	N78C	33.8, 37.5	33.0	–	59	61.0	–
	Q143C	29.6, 32.1	27.5	–	61	50.5	–

[a] The error in the PELDOR distances is 0.4 and 4 Å for principle distances 1–2 and 1-3, respectively. [b] The C_β–C_β distances of course do not allow for the length of the label and the side chain. [c] The X-ray distances are measured as the nitroxide O to O distances. [d] C_α–C_α distance.

3 matching the predictions from the crystal structure (Table 1 and the Supporting Information). As with Wza_{G58C} and Wza_{Q335C}, the peak for the 1–2 distance appears to be split and can be resolved into two distances for all sWza mutants.

We had three concerns that needed to be addressed. First, we have failed to observe the longer 1–4 and 1–5 distances. These distances of about 75 Å would have calculated modulation periods longer than 8 μs. Within the time window accessible, such long modulations are difficult to resolve from the superimposed decay caused by intermolecular spin–spin interactions.[4,5] This appears to be an experimental limitation of the approach.

Second, the PELDOR time traces reveal a modulation depth λ of only 0.4 to 0.6, whereas theory predicts $\lambda = 0.98$ for eight interacting spin labels.[8,9] Biochemical studies indicated that in solution, spin-labeled Wza and sWza are octameric, and the measured 1–2 and 1–3 distances are consistent with the octameric arrangement. The only variable that we can identify is incomplete labeling leading to a mixture of octamers, some with less than eight labels. Experiments on organic model systems have shown that in mixtures of spin oligomers the refocused echo of the detection sequence for long time windows (as in this case) is dominated by the slower-relaxing spin systems.[10] Thus, systems with fewer interacting spins would be predicted to contribute more to the echo intensity than the faster-relaxing fully occupied system. If this hypothesis is correct, then for Wza, λ is dominated by incompletely labeled Wza molecules within our timing window. Hence, shorter time windows should lead to an increase in λ. Shortening the time window for $sWza_{Q335C}$ does indeed increase λ significantly, and extrapolating the data back to $t = 0$ yields $\lambda_{t=0} = 0.94$, matching the theoretical value of 0.94 with a labeling efficiency of 85% (see the Supporting Information). It is hard to confirm the labeling efficiency independently, since mass spectrometry and chemical analysis are not accurate enough to quantify small amounts of unlabeled protein. The time-window analysis, however, identifies an experimental approach to detect and account for incomplete labeling. Rapid relaxation could also be a limitation on the signal quality in systems in which the label itself was inserted within the lipid bilayer.

Third, the presence of two peaks, a shoulder, or a broad peak at the principle distance 1–2 was puzzling. We were concerned that there was some structural artifact or an anomaly in the data acquisition or analysis. The former issue was eliminated, as sWza and labeled $sWza_{Q335C}$ are in essence identical to the full-length Wza for superimposable atoms

(see the Supporting Information). Eightfold non-crystallographic symmetry (NCS)-averaged simulated annealing 2.9 Å omit maps of $sWza_{Q335C}$ reveal strong evidence for two different conformations of the MTSSL side chain (Figure 3B). One of the conformers shows higher electron density

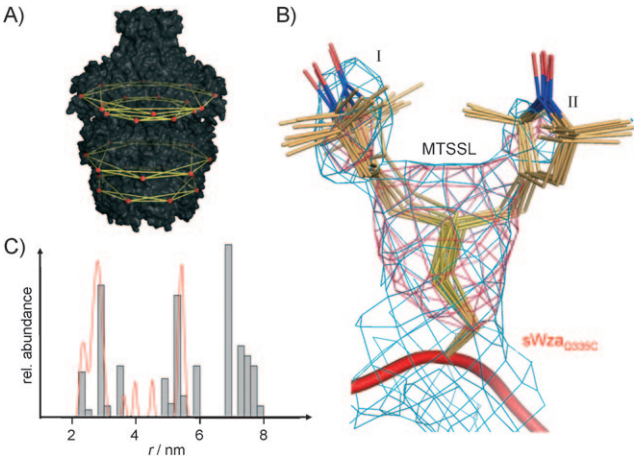

Figure 3. PELDOR distance fingerprint of Wza. A) Distances measured by PELDOR spectroscopy are indicated by yellow lines, illustrating the extended distance web over Wza (black surface). The positions of spin labels are indicated by red spheres. B) Close-up of the MTSSL label in crystallized $sWza_{Q335C}$. All of the individually refined MTSSL residues in the octameric structure have been superposed and rendered as sticks. Conformation I has been refined at an occupancy of 0.7 and conformation II at an occupancy of 0.3. The red mesh represents an eightfold NCS-averaged simulated annealing omit map contoured at 4.5 σ. The blue mesh shows the eightfold NCS-averaged 2Fo-Fc map contoured at 1.0 σ. C) Comparison between distances from PELDOR spectroscopy (red line) and X-ray structure of $sWza_{Q335C}$ (grey histogram). The histogram is corrected for the different occupancies of MTSSL conformations I and II.

(I in Figure 3B), suggesting a higher occupancy compared to the second conformer (II in Figure 3B). Conformer I is involved in several weak van der Waals interactions, mostly with hydrophobic parts of neighboring residues, whereas conformer II sticks out into the solvent. Conformations I and II were modeled with an occupancy of 0.7 and 0.3, respectively, and refined independently. These conformers are compatible with a library of MTSSL rotamers derived form MTSSL-labeled T4-lysozyme structures.[11,12] Using the crystallographic locations for the spin labels, an occupancy-weighted distance histogram was calculated, which shows an excellent fit to the PELDOR data, reproducing the splitting of the 1–2 peak (Figure 3C).

That we are capable of experimentally resolving such fine detail points to the power and potential of PELDOR spectroscopy even in complex systems such as Wza. The data are another example of discrete multiple locations of the label[11–13] rather than static or so-called smooth cone distribution.[14]

PELDOR spectroscopy has now been applied to a large, highly symmetrical membrane protein oligomer, thus expanding the demonstrated utility of the technique. We have carefully analyzed this system to quantify not only the accuracy but also the limitations of the approach. We have created a series of site-specifically labeled mutants, which provide us with a molecular fingerprint that can monitor changes in distances associated with molecular rearrangements of Wza. Thus, when combined with other structural techniques, for example, X-ray crystallography, cryo-electron microscopy, fluorescence techniques, or molecular dynamics simulations, PELDOR spectroscopy can contribute compelling molecular insights to complex biological phenomena.

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