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Pulsed Electron−Electron Double-Resonance Determination of Spin-Label Distances and Orientations on the Tetrameric Potassium Ion Channel KcsA

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Abstract: Pulsed electron−electron double-resonance (PELDOR) measurements are presented from the potassium ion channel KcsA both solubilized in detergent and reconstituted in lipids. Site-directed spin-labeling using (1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate was performed with a R64C mutant of the protein. The orientations of the spin-labels in the tetramer were determined by PELDOR experiments performed at two magnetic field strengths (0.3 T/X-band and 1.2 T/Q-band) and variable probe frequency. Quantitative simulation of the PELDOR data supports a strongly restricted nitroxide, oriented at an angle of 65° relative to the central channel axis. In general, poorer quality PELDOR data were obtained from membrane-reconstituted preparations compared to soluble proteins or detergent-solubilized samples. One reason for this is the reduced transverse spin relaxation time T_2 of nitroxides due to crowding of tetramers within the membrane that occurs even at low protein to lipid ratios. This reduced T_2 can be overcome by reconstituting mixtures of unlabeled and labeled proteins, yielding high-quality PELDOR data. Identical PELDOR oscillation frequencies and their dependencies on the probe frequency were observed in the detergent and membrane-reconstituted preparations, indicating that the position and orientation of the spin-labels are the same in both environments.

Introduction

Electron paramagnetic resonance (EPR) spectroscopy is a powerful tool for measuring long-range distances (up to 8 nm) in macromolecular systems such as polymers, oligonucleotides, and proteins.[1,2] Similar to fluorescence resonance energy transfer (FRET), which uses the electrical dipole−dipole interaction between two fluorophores, pulsed electron−electron double-resonance (PELDOR) experiments measure the magnetic dipole−dipole interaction between two paramagnetic centers to determine the distance between them.[3,4] Commonly, nitroxide spin-labels are conjugated to proteins via specifically introduced Cys residues (site-directed spin-labeling[5]). Because of the anisotropy of the hyperfine and **g** tensors of the nitroxides, only specific orientations of the nitroxide molecular axis system with respect to the magnetic field are selected, depending on the choice of the probe frequency. Therefore, only a sub-ensemble of partially ordered nitroxide molecules out of the statistical frozen sample is detected. For example, a probe pulse frequency

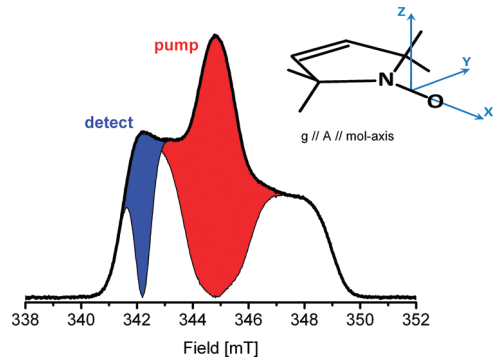

Figure 1. X-band field sweep spectrum of KcsA R64C-SL in detergent at 40 K. The pump (red) and probe (at 70 MHz offset, blue) excitation profiles are indicated. Inset: molecular axis system of the nitroxide spin-label. The **g** and **A** tensors are collinear with this axis system.

corresponding to the edge of the frozen solution nitroxide spectra at X-band frequencies, as shown in Figure 1, corresponds primarily to a selection of nitroxide molecules with the out-of-plane axis z parallel to the magnetic field. If the nitroxides are flexible and adopt all possible rotameric states,[6,7] a statistical distribution of the dipolar angle θ between the interconnecting

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(1) Schiemann, O.; Prisner, T. F. *Q. Rev. Biophys.* **2007**, *40*, 1–53.
(2) Jeschke, G. *Macromol. Rapid Commun.* **2002**, *23*, 227–246.
(3) Milov, A.; Salikov, K.; Shirov, M. *Fiz. Tverd. Tela (S.-Peterburg)* **1981**, *23*, 975.
(4) Milov, A.; Ponomarev, A.; Tsvetkov, Y. *Chem. Phys. Lett.* **1984**, *110*, 67–72.
(5) Hubbell, W.; Cafiso, D.; Altenbach, C. *Nat. Struct. Biol.* **2000**, *7*, 735–739.
(6) Sen, K.; Logan, T.; Fajer, P. *Biochemistry* **2007**, *46*, 11639–11649.
(7) Columbus, L.; Kalai, T.; Jeko, J.; Hideg, K.; Hubbell, W. L. *Biochemistry* **2001**, *40*, 3828–3846.

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vector **R** and the external magnetic field $\mathbf{B}_0$ occurs, and the length of **R** can be extracted easily from PELDOR data, for example, by using Tikhonov regularization methods.[8,9] However, if the nitroxides exhibit restricted conformational flexibility, the relative orientations of the two paramagnetic species with respect to each other and with respect to the vector **R** are not random. In such cases, because of the orientation selection induced by the probe pulses, the PELDOR modulation frequency becomes a function of both the distance and the relative orientations of the spin pair,[10,11] and current regularization methods are no longer applicable. This effect has been observed for natural paramagnetic cofactors in proteins[12] and for model bi-nitroxide compounds,[10,11,13−15] but so far has not been observed in PELDOR experiments on spin-labeled proteins. Multi-frequency cw-EPR mobility studies performed at room temperature found restricted mobility for several site-directed spin-labeled sites in lysozyme, which were rationalized by hydrophobic interactions between the spin-label and other amino acids within the protein.[16,17] Here we report on strong orientation correlations found between four spin-labels attached to the tetrameric ion channel KcsA.

Materials and Methods

KcsA Sample Preparation. Wild-type KcsA and the R64C mutant were expressed and purified as described previously[18] with a slight modification for conjugation of the spin-label (1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methyl methanethiosulfonate (MTSL; Toronto Research Chemicals). After elution of KcsA R64C from the cobalt resin, a 40-fold molar excess of MTSL was added, and the mixture was incubated at room temperature for 4 h. The MTSL reaction efficiency was estimated to be greater than 90% using a PEGylation assay. In this assay, a small amount of protein (~1 mg/mL) was incubated with a 10 mM concentration of a maleimide-conjugated 5.5 kDa polyethylene glycol polymer (PMAL; Sunbright) for 1 h at 37 °C. Exposed thiol groups that had not reacted with MTSL will react with PMAL, resulting in a ~5 kDa shift in the migration on an SDS−PAGE gel. KcsA R64C-SL (or wild-type KcsA) was then concentrated and dialyzed against a 100-fold excess of 20 mM TrisCl, pH 7.5, 150 mM KCl, and 5 mM *n*-decyl β-D-maltopyranoside (DM) overnight. The C-terminal 33 amino acids plus His-tag were removed by chymotrypsin cleavage, and the protein was further purified in the same buffer using size exclusion chromatography.

For EPR experiments, detergent-solubilized KcsA R64C-SL was concentrated, and 20% (v/v) glycerol was added for a final volume of 0.2 mL. Lipid-reconstituted KcsA R64C-SL samples were prepared using a method similar to that described previously.[19] All samples used a 3:1 mass ratio of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE; Avanti Polar Lipids, Inc.) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG; Avanti Polar Lipids Inc.). Lipids, dissolved in chloroform, were dried under an argon stream, dissolved in pentane, redried, and resuspended at 10 mg/mL in reconstitution buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM *N*-methyl-D-glucamine (NMG), 100 mM KCl, pH 7.3) using a bath sonicator. DM was added to a final concentration of 20 mM to solubilize the lipid, and the suspension was incubated for 2 h. KcsA R64C-SL (and wild-type KcsA) was added to the solubilized lipid at the desired lipid:protein ratio, and the mixture was incubated overnight at room temperature. DM was removed by dialysis against the same buffer over the course of 4 days. Liposomes, containing the reconstituted protein, were pelleted by centrifugation at 120000*g* for 2 h at 4 °C and resuspended in reconstitution buffer to a total volume of 0.2 mL. All samples contained 50 μM KcsA R64C-SL (200 μM spin-label) in 4 mm (o.d.) quartz EPR tubes (Wilmad-Labglass) and were frozen by and stored in liquid nitrogen.

PELDOR Data Collection. The dead-time free four-pulse PELDOR sequence[20] was used for all experiments. For the X-band experiments, a Bruker Elexsys E580 X-band spectrometer equipped with a PELDOR unit was used. Microwave pulses were amplified by a 1 kW TWT amplifier (ASE 117x). Typical pulse lengths were 32 ns (π/2 and π) for the probe pulses and 12 ns (π) for the pump pulse. The delay between the first and second probe pulses was varied between 132 and 196 ns in 8 ns steps to reduce contributions from proton modulations. The pulse separation between the second and third probe pulses was between 1.5 and 2.5 μs, depending on the sample preparation. The frequency of the pump pulse was fixed to the central peak of the nitroxide powder spectrum to obtain maximum pumping efficiency (Figure 1). The probe frequency was chosen 40−80 MHz above this frequency. This range corresponds to the smallest frequency offset that avoids strong pump/probe frequency overlap, and therefore large proton modulation artifacts, and the largest possible frequency offset that excites the edge of the nitroxide spectrum. Experiments at the Q-band were performed with the ELEXSYS SuperQ-FT accessory to the ELEXSYS E580 system. The cavity is a Bruker EN5107D2. The microwave power was 700 mW. The pulse length was set to 92 ns for the probe pulses (π and π/2) and 28 ns for the pump pulse; the delay between the first two pulses was set to 400 ns (plus 8 times 12 ns for hyperfine averaging). The pulse separation between the second and third probe pulses was 2.6 μs. The temperature was set to 40 K for all experiments and controlled by an Oxford flow cryostat. Typically, the signal was averaged for approximately 3 h to obtain a sufficient signal-to-noise ratio.

Simulation of PELDOR Data. An in-house MatLab simulation program, described previously,[10] was used to simulate the PELDOR time traces. For a given set of conformers, the program calculates the orientation selection function and dipolar angle distribution for the chosen pump and probe frequencies and pulse lengths and strengths. The PELDOR time traces are calculated assuming a random orientation of the molecule with respect to the external magnetic field (powder average). The simulation program was modified to account for the four spins in the C_4-symmetry-related tetramer; the final PELDOR signal is the product of the PELDOR time traces for each individual spin pair. All possible orientations of the out-of-plane z axis of the nitroxide with respect to the tetrameric plane (defined by the interspin distance vectors R_{ij}) were

(8) Jeschke, G.; Chechik, V.; Ionita, P.; Godt, A.; Zimmermann, H.; Banham, J.; Timmel, C.; Hilger, D.; Jung, H. *Appl. Magn. Reson.* **2006**, *30*, 473–498.
(9) Chiang, Y. W.; Borbat, P. P.; Freed, J. H. *J. Magn. Reson.* **2005**, *172*, 279–295.
(10) Margraf, D.; Bode, B.; Marko, A.; Schiemann, O.; Prisner, T. *Mol. Phys.* **2007**, *105*, 2153–2160.
(11) Marko, A.; Margraf, D.; Yu, H.; Mu, Y.; Stock, G.; Prisner, T. *J. Chem. Phys.* **2009**, *130*, 064102.
(12) Denysenkov, V.; Prisner, T.; Stubbe, J.; Bennati, M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13386–13390.
(13) Weber, A.; Schiemann, O.; Bode, B.; Prisner, T. *J. Magn. Reson.* **2002**, *157*, 277–285.
(14) Godt, A.; Schulte, M.; Zimmermann, H.; Jeschke, G. *Angew. Chem., Int. Ed.* **2006**, *45*, 7560–7564.
(15) Polyhach, Y.; Godt, A.; Bauer, C.; Jeschke, G. *J. Magn. Reson.* **2007**, *185*, 118–129.
(16) Sezer, D.; Freed, J. H.; Roux, B. *J. Am. Chem. Soc.* **2009**, *131*, 2597–2605.
(17) Guo, Z.; Cascio, D.; Hideg, K.; Hubbell, W. L. *Protein Sci.* **2008**, *17*, 228–239.
(18) Doyle, D. A.; Morais-Cabral, J.; Pfuetzner, R. A.; Kuo, A.; Gulbis, J. M.; Cohen, S. L.; Chait, B. T.; MacKinnon, R. *Science* **1998**, *280*, 69–77.
(19) Heginbotham, L.; LeMasurier, M.; Kolmakova-Partensky, L.; Miller, C. *J. Gen. Physiol.* **1999**, *14*, 551–60.
(20) Pannier, M.; Veit, S.; Godt, A.; Jeschke, G.; Spieβ, H. W. *J. Magn. Reson.* **2000**, *142*, 331–340.

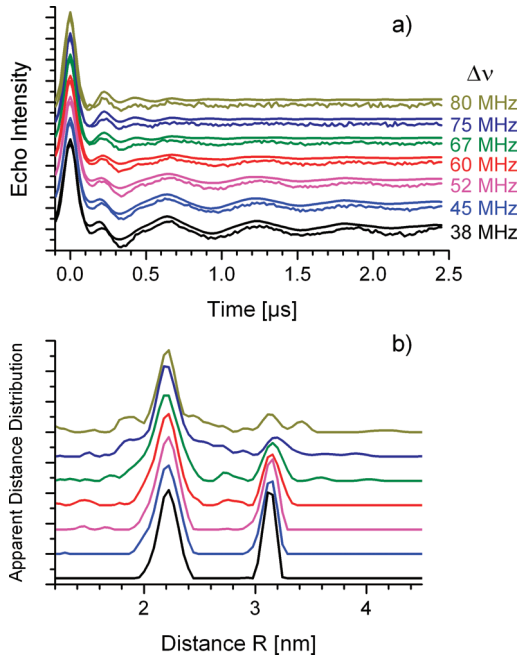

Figure 2. (a) X-band PELDOR time traces of KcsA R64C-SL in detergent and (b) apparent distance distribution obtained by the Tikhonov regularization method of the DEER Analysis program.[8] The frequency difference $\Delta\nu$ between the constant pump and variable detection frequencies ranged from 38 to 80 MHz. Simulated PELDOR time traces are plotted above the experimental data in (a). Data for the different detection frequencies are shifted relative to each other for clarity.

considered. Only the reported orientation of the spin-label gave simulation results consistent with the experimental PELDOR traces.

Results and Discussion

Figure 2a shows PELDOR time traces, acquired at a magnetic field of 0.3 T (X-band), of a frozen sample of KcsA R64C-SL solubilized in detergent at several detection frequencies. The fast and slow dipolar oscillation frequencies, arising from the short and long distances between the spin-labels in the tetramer, respectively, are clearly visible in the experimental time traces. The slow-frequency component shows a pronounced amplitude variation as a function of the detection frequency. This dependence of the PELDOR signal on the detection frequency is indicative of an angular correlation between the nitroxide spin-labels attached to KcsA.[10,20]

The variation in oscillation intensity is more clearly evident in the apparent distance distribution functions obtained by Tikhonov regularization of the PELDOR time traces (Figure 2b). The intensity ratio between the short and long distances, which theoretically should be 2:1 for the symmetric tetramer, varied experimentally from 1:1 to 5:1.

We could simulate the PELDOR time traces and their dependence on the probe frequency[10] by assuming a fixed nitroxide orientation with strongly restricted mobility (shown in Figure 2a above the experimental time traces). The deduced orientation of the nitroxide spin-labels in the KcsA tetramer is shown schematically in Figure 3: they are aligned with the N−O axes parallel to the central ion conduction pore, with the normal of the nitroxide plane at an angle β of 65° with respect to the diagonal of the tetramer and a flexibility of ±10° along the N−O bond. Only by restricting the spin-labels in this way we could explain the strong probe frequency dependence of the dipolar coupling between the two nitroxides along the diagonal direction. It is interesting to note that the three major conformations observed by crystallography for the first two dihedral angles ($\{\chi_1, \chi_2\} = \{-60°, -60°\}, \{180°, 60°\}, \{180°, -60°\}$) all produce steric clashes between the spin-label and the surrounding protein; the spin-label at position 64 is likely to have the less frequently observed negative gauche and trans conformations ($\chi_1 = -60°$; $\chi_2 = 180°$).

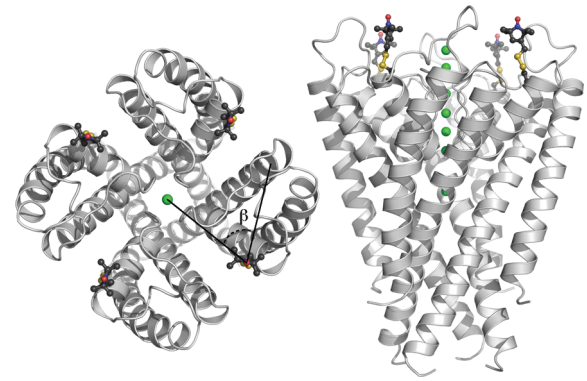

Figure 3. Spin-label orientations within the KcsA R64C-SL tetramer used for the simulations in Figure 2a. Viewed from the top (left) and side (right), the nitroxides are aligned with the N−O axis parallel to the ion conduction pore (K^+ ions within the pore are indicated by green spheres) and at an angle $\beta = 65°$ between the normal of the nitroxide plane and the direction to the channel center. The spin-label side chains are shown using ball-and-stick representations, while the rest of the protein is shown as a ribbon. This figure and Figure 5 were prepared using PyMol (DeLano Scientific LLC) and Protein Data Bank coordinates from KcsA entry 1K4C.[21]

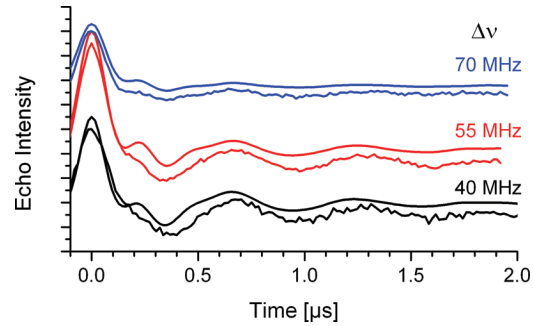

Figure 4. Q-band PELDOR time traces of KcsA R64C-SL in detergent at several probe frequencies. The frequency difference $\Delta\nu$ between the constant pump and variable detection frequencies ranged from 40 to 70 MHz. Simulated PELDOR time traces are plotted above the experimental data. Data for the different detection frequencies are shifted relative to each other for clarity.

We tested our predicted geometry of the nitroxide spin-label by performing independent experiments at a magnetic field strength of 1.2 T (Q-band). At this higher magnetic field strength, the nitroxide spectrum is no longer dominated by the anisotropic hyperfine interaction, but also has a substantial contribution from the anisotropic **g** tensor, leading to different and less intuitive orientation selection rules. We could accurately predict the Q-band PELDOR traces very well using the nitroxide orientations and flexibilities determined from the X-band PELDOR data (Figure 4), supporting our model for the nitroxide spin-label orientations in KcsA R64C-SL.

The arrangement of the spin-labels closely resembles the native Arg residues in the crystal structure of KcsA.[21] The measured nitroxide distances of 2.2 ± 0.1 and 3.1 ± 0.1 nm

(21) Zhou, Y.; Morais-Cabral, J. H.; Kaufman, A.; MacKinnon, R. *Nature* **2001**, *414*, 43.

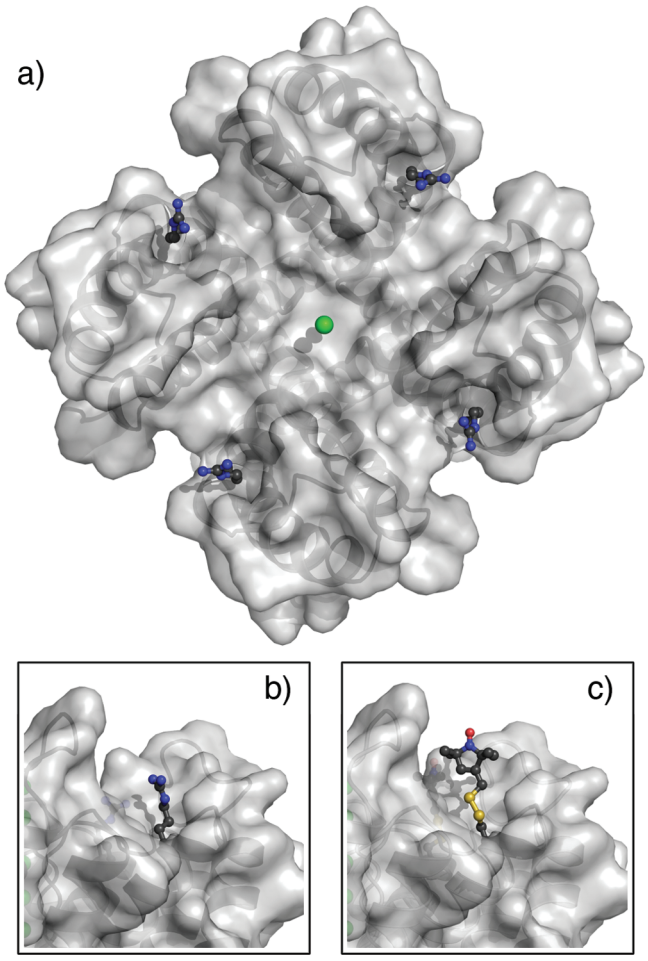

Figure 5. Structure of wild-type KcsA highlighting R64 and its local environment viewed from the top (a) and side (b). For comparison, our proposed structure of the spin-label is also shown (c). R64 and spin-label side chains are shown using ball-and-stick representations, while the rest of the protein is shown as a surface representation. K^+ ions in the central ion conduction pore are indicated as green spheres.

are in excellent agreement with the position of R64 (C^β−C^β distances are 2.2 and 3.1 nm). The R64 side chains extend upward, approximately parallel to the central channel axis. They do not make significant contact with the surrounding protein, but instead appear exposed to the solvent in a small crevice (Figure 5). Thus, the restricted mobility of the spin-label at this position is somewhat unexpected. However, it has been shown by patch-clamp single-channel conductivity measurements that E71A and R64A pore-loop mutations strongly influence the rate and extent of channel inactivation.[22] Thus, R64, located close to the selectivity filter, seems to have important structural features for the gating of this channel. Our observation of a rather rigid orientation of the spin-label might imply that the crevice is filled by non-covalently bound molecules, such as ions or lipids, which restrict the mobility of the nitroxide moiety, or that this position is structurally changed by the R64C mutation. In any case, the detection of restricted spin-label mobility correlates with changes in the inactivation of the channel observed for a mutation at this position. Therefore, the observation of a fixed orientation with restricted mobility of an attached spin-label can be used to monitor subtle details of the local surrounding of protein pockets. Indeed, cw-EPR experiments at room temperature also indicate that the mobility of the nitroxide spin-labels at this position is strongly restricted (Figure 6), supporting our PELDOR analysis.

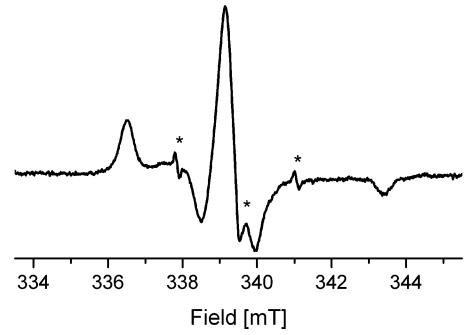

Figure 6. Room temperature cw-X-band EPR spectrum of KcsA R64C-SL solubilized in detergent. Lines marked with an asterisk are due to residual free spin-labels (not bound to protein).

Table 1. Transverse Relaxation Times (T_2) for KcsA R64C-SL Samples

KcsA sample[a]	preparation[b]	T_2 (μs)
50 μM R64C-SL	DM/20% glycerol	1.8 ± 0.1
50 μM R64C-SL	1:10 PE/PG	0.7 ± 0.1
50 μM R64C-SL	1:80 PE/PG	0.8 ± 0.1
50 μM R64C-SL + 50 μM WT	1:10 PE/PG	0.8 ± 0.1
50 μM R64C-SL + 150 μM WT	1:10 PE/PG	1.3 ± 0.1

[a] The concentration indicated is that of the tetrameric channel. [b] For lipid samples, the ratio indicated is total protein:total lipid by mass.

We performed X-band PELDOR experiments on frozen KcsA R64C-SL samples reconstituted in a 3:1 mass ratio mixture of POPE and POPG (PE/PG) lipids to determine whether these structured nitroxides persist in a native membrane environment. We initially prepared liposomal samples at a 10:1 lipid:protein ratio (mass), but could not detect any dipolar modulation of the PELDOR signal. Indeed, the transverse spin relaxation time T_2 of the nitroxides in this sample (0.7 ± 0.1 μs) was much lower compared to that of detergent-solubilized KcsA R64C-SL (1.8 ± 0.1 μs). To accurately detect distances in the 2−6 nm range, the pulse separation in the PELDOR experiment should be at least 2 μs, such that a T_2 greater than ∼1 μs is required for adequate signal intensity.

In liposomal samples, KcsA is restricted to the lipid bilayer and is thus able to occupy a much smaller volume compared to KcsA solubilized in detergent. This leads to a higher local concentration of spin-labeled tetramers within the membrane, resulting in decreased T_2 due to enhanced dipolar relaxation between nitroxides. To reduce this crowding effect, we reconstituted KcsA R64C-SL at larger lipid to protein ratios (up to 80:1), but were only able to obtain marginally increased T_2. However, when diamagnetic (unlabeled) wild-type KcsA was added to the KcsA R64C-SL reconstitution, a significantly increased T_2 was observed (Table 1). This requirement for unlabeled KcsA suggests that protein aggregation is the likely cause for the reduced T_2 in liposomal samples; the additional tetramers act as spacers to increase the average distance between spin-labeled molecules, thereby reducing dipolar relaxation. Similar effects were observed for the membrane transport protein

(22) Cordero-Morales, J. F.; Cuello, L. G.; Zhao, Y.; Jogini, V.; Cortes, D. M.; Roux, B.; Perozo, E. *Nat. Struct. Mol. Biol.* **2006**, *13*, 311–318.

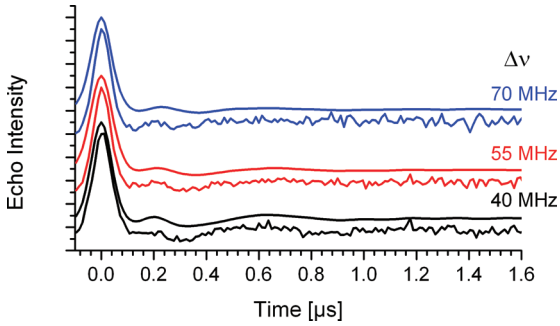

Figure 7. X-band PELDOR time traces of liposomal KcsA R64C-SL reconstituted with 3-fold excess wild-type KcsA into PE/PG at a 10:1 lipid to total protein mass ratio. Simulated time traces (shown above the experimental data) are based on the nitroxide positions in Figure 3.

BtuB by dilution with diamagnetic non-labeled protein.[23] No exchange between labeled and non-labeled protomers between the KcsA tetramers occurred, as a constant PELDOR modulation depth was observed.

Our optimized lipid preparations consisted of a 1:3 molar ratio of KcsA R64C-SL to wild-type KcsA with a lipid to total protein ratio of 10:1 (mass) and had a T_2 of 1.3 ± 0.1 μs. This yielded PELDOR time traces of a quality similar to that from detergent samples, with comparable modulation depths and identical oscillation frequencies (Figure 7). Again, the amplitude of the slow-frequency component was significantly affected by the detection frequency offset, and the PELDOR data could be correctly described using the orientation parameters obtained from the detergent sample. Thus, the oriented nitroxide spin-labels, and hence the local protein environment, are preserved between the detergent and lipid samples.

(23) Xu, Q.; Ellena, J. F.; Kim, M.; Cafiso, D. S. *Biochemistry* **2006**, *45*, 10847–10854.

Conclusion

We have shown for the first time that strong angular correlations between nitroxide spin-labels attached to a protein can occur. Performing PELDOR experiments at several detection frequencies permitted the separation of orientation and distance information, and enabled us to determine the spin-label orientations in the tetrameric ion channel KcsA R64C-SL. We have also shown that the reduced T_2 in membrane-reconstituted samples can be overcome by the addition of unlabeled protein to the reconstitution. In the case of KcsA R64C-SL reconstituted in lipids, crowding of tetramers resulted in a shorter T_2 and a reduced PELDOR modulation depth, which strongly limited the quantitative comparison of detergent and lipid data. Reconstituting mixtures of spin-labeled paramagnetic and unlabeled diamagnetic tetramers efficiently suppressed this effect, and high-quality PELDOR data could be obtained. The identical oscillation frequencies and their dependencies on the detection frequency offset observed in the detergent and liposomal preparations indicate that the position and orientation of the spin-labels are the same in both environments.

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