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# Orientation selective DEER measurements on vinculin tail at X-band frequencies reveal spin label orientations

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ABSTRACT

Double electron electron resonance (DEER) spectroscopy has been established as a valuable method to determine distances between spin labels bound to protein molecules. Caused by selective excitation of molecular orientations DEER primary data also depend on the mutual orientation of the spin labels. For a doubly spin labeled variant of the cytoskeletal protein vinculin tail strong orientation selection can be observed already at X-band frequencies, which allows us to reduce the problem to the relative orientation of two molecular axes and the spin–spin axis parameterized by three angles. A full grid search of parameter space reveals that the DEER experiment introduces parameter-space symmetry higher than the symmetry of the spin Hamiltonian. Thus, the number of equivalent parameter sets is twice as large as expected and the relative orientation of the two spin labels is ambiguous. Except for this inherent ambiguity the most probable relative orientation of the two spin labels can be determined with good confidence and moderate uncertainty by global fitting of a set of five DEER experiments at different offsets between pump and observer frequency. The experiment provides restraints on the angles between the *z* axis of the nitroxide molecular frame and the spin–spin vector and on the dihedral between the two *z* axes. When using the same type of label at both sites, assignment of the angle restraints is ambiguous and the sign of the dihedral restraint is also ambiguous.



## 1. Introduction

Double electron electron resonance (DEER) spectroscopy, also known under the name of pulsed electron double resonance (PELDOR) has become a routinely used method to determine inter spin distances between spin labels bound to protein molecules [1,2]. Early on it was established both experimentally and theoretically that DEER data depend on the mutual orientation of the spin label molecules [3]. The concept experienced a renaissance with the advent of high field DEER, where this dependence is stronger [4–7]. However, useful information can also be extracted at the more common X-band frequencies, provided that the labels are sufficiently rigid [5,8–10]. Spin labels typically used in proteins, such as methanethiosulfonate spin label (MTSSL), are inherently flexible and orientation correlation is often negligible even at high fields. However, if spatial freedom of the spin label side chain is strongly restricted by interactions with the protein backbone and neighboring side chain atoms, only a few possible rotameric states may be populated. In extreme cases, a single rotamer may dominate and if this happens at both labeled sites, strong correlation of the orientations of the two spin label side chains arises. Distance distributions calculated from the DEER spectra under the assumption of an isotropic distribution of orientations may then lead to misinterpretation and averaging of the orientation selection effect by acquisition of data over a range of observer fields [11] may be advisable. If the data at the different observer fields are separately stored they can be used for proper analysis of relative spin label orientation, which could in turn further improve the quality of the distance distribution, since the averaging generally is not complete [12]. The information on relative spin label orientation is of interest for characterizing conformational changes of proteins that are accompanied by reorientations of the spin label side chains.

Relative orientations of paramagnetic centers can be determined by orientation selective DEER [3,4,6,8–10]. The hyperfine coupling anisotropy of the commonly used nitroxide spin labels enables orientation selectivity by shifting pump and observer pulse frequencies within the corresponding range of the EPR absorption

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spectrum. Measurements on nitroxide biradical model compounds at X- and W-band frequencies have been reported to show orientation selectivity and the spin pair geometry for these compounds could be revealed [5,6,8]. Furthermore, orientations between two radical pairs within bacterial photosynthetic reaction centers have been determined using this method [13]. Additionally, spin-labeled flavoprotein reductase/reduced iron–sulfur ferredoxin protein complex and a bi-radical with two Cu(II) ions have been shown to provide orientation information of the radical centres [14]. In a recent PELDOR study on the spin labeled tetramer of the potassium ion channel, KcsA, the orientation of the attached four spin labels could be determined under the assumption of $C_4$-symmetry [15]. In all mentioned cases the simulations of the nitroxide DEER spectra were based on molecular models defining the orientations of the spin labels, which were then validated by experiments. Such approaches can demonstrate that a molecular model is consistent with experimental data but not whether the solution is unique.

Two studies of Marko et al. address the problem of inferring information without a specific model of the system under investigation. The first of those studies [9] provides an algorithm for directly obtaining an orientation intensity function $\lambda(\Theta_{dd})$, where $\lambda$ is the modulation depth and $\Theta_{dd}$ the angle between the spin–spin vector and the static magnetic field direction, which can be applied for uncorrelated distance and orientation distributions. The orientation intensity function $\lambda(\Theta_{dd})$ in turn can be computed from the relative orientation of the spin labels, but no algorithm has yet been derived for the inverse problem of computing relative orientation from $\lambda(\Theta_{dd})$. The second study by Marko et al. provides analytical expressions for directly computing relative orientation in the special case where the problem can be posed in terms of only one angle $\beta$ between the spin–spin vector and the coinciding unique axes of the nitrogen hyperfine tensor of the two labels [10]. Whereas this special case can be assumed for certain spin labels in DNA it does not apply to pairs of spin labels in proteins.

In this work we address the problem of possible ambiguity in determination of relative spin label orientations. We applied X-band orientation selective DEER measurements on spin labeled double mutants of the cytoskeletal protein vinculin tail using five different frequency offsets between pump and observer pulses. In contrast to high-field DEER this approach allows for neglecting g tensor non-axiality and thus reduces the parameter space that needs to be scanned for solutions (see also [10]). The obtained DEER spectra were analyzed by calculating the corresponding Pake patterns (Fourier transform of the background corrected DEER spectra) in dependence of mutual spin label $z$-axis orientations for a complete parameter grid and relating them to the experimental data by means of the root mean square deviation (RMSD) between the simulated and experimental Pake spectra for all orientations. The thus obtained orientation profile reveals the most probable mutual orientation of the spin label side chains. In this approach it is assumed that the two R1 side chains, separated by a defined distance, exhibit such a narrow distribution of mutual orientations that the problem can be posed in terms of a mean relative orientation.

This paper is organized as follows. The Section 3 specifies our assumptions and approximations and describes the reduction of parameter space and the complete parameter grid to be searched to find all possible solutions of the problem. The Section 4 describes the experimental strategy used for obtaining orientation-dependent DEER data at X-band frequencies, discusses the primary results and dipolar spectra in a model-independent way and reports our finding that effective symmetry of the spin system for computing DEER data is higher than symmetry of the spin Hamiltonian. A list of symmetry-related triples of Euler angles for the present case is provided. We conclude with a critical assessment of the information content of orientation-selective DEER measurements.

## 2. Materials and methods

### 2.1. Sample preparation

Vinculin endcoding cDNAs used in this study were obtained as described by Chandrasekar et al. [16]. Vinculin tail (Vt) constructs in pQE-30 (Qiagen) encoded amino acids 858–1066, are equipped with an N-terminal FLAG and His tag. The Quick Change® Method (Stratagene) was used to replace the three cysteines at positions 950, 972 and 985 with alanine. Subsequently, the triple alanine mutant was used to generate the double mutant Vt-A901C/A957C. The mutant construct was verified by DNA sequencing. The recombinant double cysteine mutant was expressed and purified as described by Ziegler et al. [17]. Spin labeling with the (1-oxy-2,2,5,5-tetramethyl-pyrrolinyl-3-methyl)methanethiosulfonate spin label (MTSSL) (TRC, Alexis Biochemicals) was carried out overnight by incubating the protein bound to Ni–NTA beads (Qiagen) for 12–16 h with 2 mM MTSSL. Unbound label was removed by washing the Ni–NTA beads several times with 50 mM phosphate buffer, pH 7.2. The spin labeled proteins were eluted according to the manufacturer's instructions (Qiagen) and transferred into 20 mM phosphate buffer (80 μM EGTA, pH 7.2) using PD10 desalting columns (GE Healthcare). Protein integrity was confirmed using coomassie-stained SDS–PAGE gels and circular dichroism spectroscopy. Protein concentrations were determined using a BCA assay (Thermo Scientific). Size exclusion gel filtration chromatography experiments have shown that Vt is monomeric at room temperature. DEER experiments on singly labeled Vt variants in aqueous buffer solutions have provided evidence for a small fraction (<30%) of dimeric Vt present in the sample [18]. For orientation selective DEER measurements the double labeled Vt, Vt901R1/957R1, was mixed with unlabeled wild type Vt (Wt) in a molar ratio of ratio 1:2 (Vt:Wt) in order to suppress contributions of intermolecular spin–spin interaction to the DEER traces.

### 2.2. Pulse EPR spectroscopy (DEER)

Pulse EPR experiments (DEER) were performed at X-band frequencies (9.4 GHz) with a Bruker Elexsys 580 spectrometer equipped with a Bruker Flexline split ring resonator ER 4118XMS3. 40 μl of sample solution at a final protein concentration of 80–120 μM containing 10% of deuterated glycerol was filled into an EPR quartz capillary (2 mm inner diameter). A continuous flow helium cryostat (ESR900; Oxford Instruments) and an Oxford Instruments ITC 503S were used for temperature controlling. All measurements were performed using the four-pulse DEER sequence: $\pi/2(\nu_{obs}) - \tau_1 - \pi\ (\nu_{obs}) - t' - \pi\ (\nu_{pump}) - (\tau_1 + \tau_2 - t') - \pi\ (\nu_{obs}) - \tau_2 - echo$ [2]. For the DEER pulses at the observer frequency the $\langle x\rangle$ channels were used. A two-step phase cycling $(+\langle x\rangle, -\langle x\rangle)$ was performed on $\pi/2(\nu_{obs})$. Time $t'$ is varied, whereas $\tau_1$ and $\tau_2$ are kept constant, and the dipolar evolution time is given by $t = t' - \tau_1$. Data were analyzed only for $t > 0$. The resonator was overcoupled to $Q \sim 100$.

For the standard DEER experiment to extract the mean distance, the pump frequency, $\nu_{pump}$, was set to the center of the resonator dip and coincided with the maximum of the nitroxide EPR absorption spectrum, whereas the observer frequency, $\nu_{obs}$, was 65 MHz higher and coincided with the low field local maximum of the absorption spectrum with observer pulse lengths of 16 ns for $\pi/2$ and 32 ns for $\pi$ pulses and a pump pulse length of 12 ns. Deuterium modulation was averaged by adding traces at eight different $\tau_1$ values, starting at $\tau_{1,0} = 400$ ns and incrementing $\tau_1$ by $\Delta\tau_1 = 56$ ns. All measurements were performed at a temperature of 50 K. Background correction of the DEER spectrum was carried out assuming a homogeneous 3D background. Interspin distance