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Inter-spin distance determination using L-band (1-2 GHz) non-adiabatic rapid sweep electron paramagnetic resonance (NARS EPR)

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Abstract

Site-directed spin-labeling electron paramagnetic resonance (SDSL EPR) provides insight into the local structure and motion of a spin probe strategically attached to a molecule. When a second spin is introduced to the system, macromolecular information can be obtained through measurement of inter-spin distances either by continuous wave (CW) or pulsed electron double resonance (ELDOR) techniques. If both methodologies are considered, inter-spin distances of 8 to 80 Å can be experimentally determined. However, there exists a region at the upper limit of the conventional X-band (9.5 GHz) CW technique and the lower limit of the four-pulse double electron-electron resonance (DEER) experiment where neither method is particularly reliable. The work presented here utilizes L-band (1.9 GHz) in combination with non-adiabatic rapid sweep (NARS) EPR to address this opportunity by increasing the upper limit of the CW technique. Because L-band linewidths are three to seven times narrower than those at X-band, dipolar broadenings that are small relative to the X-band inhomogeneous linewidth become observable, but the signal loss due to the frequency dependence of the Boltzmann factor, has made L-band especially challenging. NARS has been shown to increase sensitivity by a factor of five, and overcomes much of this loss, making L-band distance determination more feasible [1]. Two different systems are presented and distances of 18–30 Å have been experimentally determined at physiologically relevant temperatures. Measurements are in excellent agreement with a helical model and values determined by DEER.

Keywords

EPR; ESR; NARS; non-adiabatic rapid sweep; distance determination

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1. Introduction

Site-directed spin-labeling electron paramagnetic resonance (SDSL EPR) has emerged as a useful biochemical tool over the last three decades because of its versatility and specificity [2, 3]. The application of this method in various EPR experiments allows investigation into the localized structure and motion of a stable paramagnetic probe attached to a cysteine strategically mutated into a protein. When a second probe is added to the system, macromolecular information can be obtained through measurement of inter-spin distances either by conventional continuous wave (CW) techniques or by pulsed electron double resonance (ELDOR) techniques. Such measurements can be used as constraints in protein structure calculations, which are vitally important to understanding protein function.

When a system is not motionally averaged, two spins are coupled to each other by a through-space dipolar interaction, which causes splitting of the resonance lines. The extent of this splitting is inversely related to the cube of the distance, r , as shown in Eq. (1), where D is the splitting of the signal in Gauss, g is the electron Zeeman factor, and β is the Bohr magneton.

$$D=3g\beta/2r^3 \quad (1)$$

Rabenstein and Shin showed that the dipolar broadened CW spectrum is the sum of the two single-label spectra convolved with a dipolar broadening function consisting of a Gaussian distribution of Pake doublets [4, 5]. Thus, Fourier deconvolution can be used to isolate the broadening function and calculate the inter-spin distance and distribution width from experimental spectra.

One major limitation of the CW method is that it is only applicable at distances where the energy of interaction is sufficiently large enough to broaden the spectrum. At X-band (9.5 GHz), where conventional EPR is performed, g -anisotropy dominates the rigid limit spectrum, leading to linewidths ranging from 6 to 8 G. As a result, the upper limit for distance determination based on Eq. (1) lies between 16 to 18 Å depending on the inhomogeneous linewidth of the single label.

Pulsed ELDOR techniques overcome this limitation through pulse sequences that allow observation —inside the inhomogeneous linewidth. The most common pulse sequence used to measure inter-spin distances in SDSL EPR is four-pulse double electron-electron resonance (DEER) [6]. DEER observes the modulatory effects the relaxation one set of spins has on the spin echo of another. The frequency and depth of this modulation can be related to the inter-spin distance and distribution.

Like CW, DEER has some limitations. It must be performed at liquid nitrogen temperatures to maximize phase memory time (T_m). Values of T_m vary, placing the upper limit of DEER anywhere from 50 to 80 Å depending on the sample. Recent studies have shown that deuteration of the solvent and peptide backbone can improve this range considerably [7]. Nevertheless, measuring inter-spin distances at liquid nitrogen temperatures may not represent physiological conditions, since the stability of different conformations may be more favorable at lower temperatures. Similarly, DEER is typically performed in the presence of cryo-protectants such as polyethylene glycol (PEG) or glycerol. These solutes are osmolytes and can have a stabilizing or destabilizing effect on proteins. This can sometimes be beneficial, particularly for protein crystallization, but they can also alter the native structure of some proteins and prevent conformational changes in others [8–11].

The lower limit of DEER is determined by the bandwidths of the pump and observed pulses. To avoid the presence of spurious signals, they must be greater than the dipolar coupling frequency. As a result, the lower limit of DEER under optimal conditions is in the 16 to 20 Å range. Recent work by Banham *et al.* has provided a way to correct measurements at the lower limit, but they also concluded that to obtain a complete picture of the dipolar interaction, CW and DEER data should be combined [12]. Consequently, there remains a desire to develop new methods that fill this gap. The recently developed double quantum coherence (DQC) is suited to perform measurements in this range, but it also requires liquid nitrogen temperatures to maximize T_m [13, 14]. To avoid these drawbacks, the next logical step is to improve the CW technique so that room temperature measurements can be made in this range.

Simulations by Antsiferova and Lebedev of a nitroxide at 1 GHz showed that at the rigid limit, the $M_I = 0$ line is dominated by g -anisotropy as at X-band, but the residual Zeeman anisotropy is just 0.5 G because of the change in frequency [15]. These simulations assumed optimal conditions where no additional broadening mechanisms are considered. Recent work by our group shows that under typical experimental conditions, a linewidth of 2 G is realistic [16]. The narrow central feature is an ideal marker to measure inter-spin distances using CW EPR.

A significant drawback to using L-band is the factor of 5–10 reduction in signal due to the frequency dependence of the Boltzmann factor relative to X-band. This loss is somewhat compensated when observing the $M_I = 0$ line because of reduced Zeeman broadening and is further compensated by lower dielectric losses. Nevertheless, in our hands, the sensitivity at L-band using standard instrumentation was not sufficient to measure distances reliably. In the authors' recent publication, a new CW method was developed that improved sensitivity by at least a factor of five [1]. This advance made distance determination using L-band feasible.

Non-adiabatic rapid sweep (NARS) EPR consists of repetitively sweeping the magnetic field linearly through a spectral fragment at a rate that is sufficiently high to overcome receiver noise, microwave phase noise, and environmental microphonics, but is sufficiently slow and at low enough microwave power that adiabatic rapid passage is avoided. This allows the spin system to remain near thermal equilibrium. The use of NARS not only increases the sensitivity, but provides a pure absorption response since magnetic field modulation is not needed.

Field modulation is used in the conventional CW experiment to overcome receiver noise and source noise by offsetting the signal from the carrier frequency. However, field modulation of any amplitude causes spectral broadening and inherently sacrifices a significant amount of signal. Figure 1 displays this phenomenon using pseudomodulation, a technique developed by Hyde *et al.* that precisely mimics field modulation [17]. To obtain the maximum range of measureable distances, <1% spectral broadening resulting from field modulation can be tolerated. Under these conditions, more than 95% of the signal is given up. This makes the conventional L-band CW experiment especially unappealing.

The work presented here shows that by using NARS, the central feature of an L-band EPR spectrum can be used reliably to measure inter-spin distances from 15 to 30 Å at physiologically relevant temperatures. As with X-band, the upper limit of this technique will vary depending on the signal-to-noise ratio (SNR) and inhomogeneous linewidth of the single label-spectrum.

2. Methods

2.1. Helical peptides

A series of α -helical peptides of the form Ac-(AAAAK)₄A-NH₂ with cysteines strategically placed throughout were synthesized by and purchased from the Protein and Nucleic Acid Core Facility at the Medical College of Wisconsin (Milwaukee, WI). This series was chosen specifically because of previous use by other groups as a ruler for EPR distance determination [4, 12]. Each peptide was further purified using high-pressure liquid chromatography to eliminate excess salts, lyophilized, and then reconstituted in 100 mM HOAc, pH 5. The α -helical nature of the peptides was confirmed using circular dichroism. A five-fold molar excess of deuterated (1-oxy-2,2,5,5,-tetramethylpyrroline-3-methyl) methanethiosulfonate (pdMTSL) (Toronto Research Chemicals, Toronto, ON, Canada) was added, and the pH was adjusted to 7. The mixture was allowed to react overnight at 4°C. Excess spin label was removed using an SP Sepharose cation exchange column, followed by a C18 reverse phase cartridge to remove the GdnHCl needed to elute the peptide from the cation exchange column. The peptides were then lyophilized and reconstituted in a 50 mM MOPS pH 7.1 buffer to an approximate concentration of 250 μ M. Motional averaging of the spectrum was avoided by adding 50% v/v deuterated glycerol and collecting spectra at -30°C. For brevity, the peptides will be designated as PA-X, where X represents the location of the cysteine.

2.2. T4 lysozyme

Plasmids for five single cysteine mutants and three double cysteine mutants of T4L were kindly provided by Dr. Wayne Hubbell (University of California, Los Angeles). All mutants were expressed, purified, and spin-labeled as previously described [18]. Briefly, a single colony transformant of *E. coli* BL21 containing the T4L plasmid was grown overnight in ~10 mL LB medium containing 100 μ g/mL ampicillin at 37°C and used to inoculate 1 L of LB medium containing 100 μ g/mL of ampicillin. The 1 L culture was grown at 37°C to an OD₆₀₀ of 1.0–1.2, induced with 1 mM IPTG, and allowed to grow for an additional 1.5 hours before harvesting by centrifugation. Cell pellets were resuspended in a minimal amount of lysis buffer (25 mM Tris/MOPS, 0.1 mM EDTA, pH 7.6) and sonicated for 5 min on ice (standard flat disruption horn, 35% output, duty cycle 4). The resulting cell lysate was centrifuged at 30,000 g at 4°C for 30 min, and the soluble fraction was loaded onto a HiTrap™, SP Sepharose HP cation exchange column (GE Healthcare) equilibrated with lysis buffer containing 5 mM DTT. A 1 M NaCl gradient was used to elute T4L, and SDS-PAGE was used to verify its location and purity.

Purified T4L mutants were spin-labeled by passing them over a HiPrep™ 26/10 desalting column (GE Healthcare) equilibrated with 50 mM MOPS and 25 mM NaCl, pH 6.8; diluting to approximately 10 μ M; and reacting with a 10-fold molar excess of pdMTSL. The reaction was allowed to continue overnight at 4°C. Excess spin label was removed using the same desalting column and concentrated to approximately 500 μ M using an Amicon Ultra 10 K centrifugal filter. Deuterated glycerol was added (50% v/v) for long-term storage and prevention of motional averaging.

2.3. EPR spectroscopy

EPR spectra were collected using a Varian E-9 spectrometer fitted with an L-band bridge of in-house design equipped with a B-H15 Bruker field controller and a one-loop-one-gap resonator operating at 1.9 GHz with a 70 μ L active region. Temperature control was achieved with a modified Varian V6040 variable temperature control unit equipped with an Omega microprocessor and gas exchange system. Samples were not deoxygenated. NARS was performed using the setup described previously with one exception [1]: a new voltage-

controlled amplifier of in-house design was implemented, allowing field excursions of 50 G peak-to-peak on the triangular waveform. The frequency of the triangular waveform was adjusted to 2.6 kHz (260 kG/s).

The nitroxide spectrum in its entirety was collected in segments because of concern about potential non-linearities in the sweep due to eddy current formation on the resonator walls. Each 50 G segment was averaged 400 k times for T4L and 100 k for the peptide (154 and 38.5 s/segment and 72 and 18 min/spectrum, respectively). After completion of each segment, the static field was stepped 5 G, and the collection was repeated. This process was performed over 140 G. Spectra were pieced together by isolating the central 5 G of each segment and concatenating them end to end. Although no change was visible when 10 G fragments were concatenated, the former was chosen to ensure accuracy. The baseline was removed using a third-degree polynomial fit. Baseline offsets arise from the magnetic field dependence of Lorentz forces. Spectra were further processed using a Fourier filter to remove high frequency noise as well as the periodicity arising from the concatenation process.

2.4. Dipolar analysis

Inter-spin distances were determined using a software program used previously by Hustedt et al. [19]. The program uses least-squares analysis to determine the best fit to the double-label spectrum resulting from a single-label spectrum convolved with a set of different dipolar broadening functions—namely, a Pake function averaged over a Gaussian distribution of distances. A deconvolution approach is thought to be directly equivalent. Because the program uses a Levenberg-Marquardt algorithm, several different starting points were used to ensure an accurate fit. Measurements were performed in the central 15 G of the spectrum where the largest spectral changes were expected.

3. Results

The upper limit for the L-band NARS technique is dependent on the SNR and the inhomogeneous linewidth, which is affected by several factors. These include residual g-anisotropy, unresolved superhyperfine structure of the protons, or deuterons in this case, of the spin probe, and coordination of the spin probe to the peptide backbone or solvent nuclei to name a few. Because of these interactions, the linewidths of the T4L mutants (1.9 G peak-to-peak of the first harmonic) differed from those of the helical peptides (2.3 G). The reason for the difference lies in the need for higher viscosities for the peptide to avoid averaging of the dipolar vector. At -30°C the viscosity of a 50% v/v glycerol-water mixture increases by approximately an order of magnitude relative to room temperature. The lower temperature creates an environment where the spin probe can interact with solvent nuclei, broadening the line slightly. This interaction is averaged out at room temperature for the T4L mutants, which are larger in size and do not require higher viscosities.

Figure 2 displays the pure absorption spectra of PA-3 and all of the peptide double mutants normalized to spin concentration. The intensity of the central feature is diminished in all of the double mutants relative to the single mutant, which is indicative of spectral broadening. Analysis of the spectra by the convolution program showed that the broadening could solely be accounted for by the dipolar interaction. The distances and distributions responsible for these broadenings, as determined by least-squares analysis of the fit, can be found in Table 1.

The quality of fit was evaluated by calculating the least-squares value over a wide range of distances and distributions (data not shown). The measured distances and distributions were identified by a minimum least-squares value. In all cases, a distinct minimum was present

when distance was varied. However, when the distribution width was probed, clear minima were present in PA-3,11, PA-3,12, and PA-3,18, but not in PA-3,13 or PA-3,14. In these two cases, the least-squares values suggested a distribution width greater than 4 Å.

The accuracy of the measurements was assessed by comparing the experimental values to those calculated by Banham et al. using a helical model [12]. Table 1 shows that the L-band NARS measurements are in excellent agreement with the model. The largest deviations are observed at the shortest and longest distances. At shorter distances, the energy of interaction is at its greatest, causing the most spectral broadening. Because the intensity of the central feature is greatly diminished, the shoulders dominate the spectrum. Consequently, the least-squares fit is dependent on the fitting of the slopes of the shoulders instead of the shape of the central feature. At longer distances, the spectral broadening becomes small relative to the intrinsic linewidth. This change is difficult to quantify accurately and is also dependent on signal to noise. The same problem is encountered at the upper limit of the CW X-band technique.

The distribution profiles of the experimental values and the helical model are also in relatively good agreement. When a distinct minimum in the least-squares analysis is present (PA-3,11, PA-3,12, and PA-3,18), the experimental measurements are within 1 Å of the expected value. The measurements that lacked least-squares minima suggested distributions greater than 4 Å, which is also in agreement, suggesting L-band is suitable to measure both the distance and distribution.

Figure 3 displays the pure absorption spectra of T4L-68 and three double mutants normalized to spin concentration. These mutants were specifically chosen because they spanned the entire range of distances where broadening could be expected with the intrinsic linewidth of the T4L single mutant. The expected distances were determined by DEER performed by Fleissner et al. and are shown in Table 2 with calculated distances using L-band NARS EPR [20]. The measurements are in good agreement with each other, though there are some minor deviations, which are likely due to the different conditions under which the two experiments were performed.

To ensure the quality of the measurement, least-squares analysis was performed as before. Minima were present in the analysis of all three double mutants when the distance was varied, but the least squares value for T4L-68/109 did not change from $r = 29\text{--}33$ Å. This is either a result of being at the upper limit of the method or an averaging of the dipolar interaction since the rotational correlation time is just fast enough in this environment. Distinct minima were present for all three mutants when the distribution width was changed.

4. Discussion

The work presented here shows that L-band EPR can be used to increase the upper limit of CW distance determination. The extent of this expansion will vary depending on the linewidth of each single mutant. Two different soluble systems have been presented, both in the presence of 50% deuterated glycerol. These conditions differently affected the range of distances available for measurement in each system, but were used to reasonably mimic the conditions of the DEER experiment. As a result, the conditions were not optimal for L-band CW distance determination. Ideally, the experiment should be performed at room temperature and in the presence of a non-osmolyte such as Ficoll™ to avoid averaging the dipolar vector and provide the most physiologically relevant conditions possible. The added benefit of Ficoll™ is it produces narrower lines relative to glycerol at non-freezing temperatures [16]. If the use of glycerol cannot be avoided, D₂O should be used instead of H₂O, especially at lower temperatures where broadening by solvent nuclei is apparent. The

reader is referred to work by Lopez *et al.* for further discussion on the effects of different cosolvents on spin label motion and protein structure [21].

Broadening resulting from the unresolved hydrogen superhyperfine structure of MTSL and solvent nuclei coupling has already been considered in the work here by deuterating the glycerol and spin label. However, several other environmental factors contribute to linewidth and diminish the range of measurable distances. For instance, collisions with molecular oxygen may broaden lines a few tenths of a Gauss. Therefore, deoxygenation could increase the range by lessening Heisenberg exchange between molecular oxygen and the spin label. In the case of buried residues, lines could broaden if the spin probe coordinates with the peptide backbone. Such an effect can potentially be reduced by deuterating the protein. No matter the situation, the single-label spectrum should always be analyzed first to identify the upper distance limit of a particular mutant, and identify the conditions under which the greatest range of distances is measurable.

One potential disadvantage of the L-band technique that emerged here is the possible inability to determine distribution profiles via the lineshape change. To identify where the effects of distribution width are most prominent, absorption spectra of PA-3 broadened by four different inter-spin interactions with varying distribution widths were simulated (Fig. 4). Interestingly, the distribution width alters the spectrum differently depending on the inter-spin distance. As the distribution width is increased at $r = 17.5 \text{ \AA}$, the central feature narrows with the addition of longer distances, while the intensity of the shoulders increases with the addition of shorter distances. A similar trend is observed at $r = 20 \text{ \AA}$, though the changes are less dramatic, as shown by the residuals. The trend appears to shift at $r = 22.5 \text{ \AA}$. Instead of the central feature narrowing as the distribution width increases, broadening is observed. This occurs because the longer distances added with wider distributions are narrower than the inhomogeneous linewidth. Thus, shorter distances are more heavily weighted in the spectrum.

The transition between the two trends is conveniently located at the distances where the experimental measurement of the distribution measurements is unclear. The changes determined by distribution width at $r = 20 \text{ \AA}$ are very small, particularly between 3 to 5 $\text{\AA}$ where the intensity of the central feature changes very little. This partly explains why the least-squares analysis of PA-3,13 and PA-3,14 lacks uniqueness. Nevertheless, quantitative measurements are feasible, and in every case, qualitative information can be gathered, quelling any concern about measuring distribution profiles.

L-band distance determination becomes more advantageous when the angular terms in the dipolar interaction are considered. Hustedt et al. showed that each of the six angles in the dipolar vector affect the broadening of the spectrum differently [22]. The result is a somewhat asymmetric broadening of the X-band spectrum since the g-anisotropy is well resolved. This can lead to poor estimation of the distribution width when using the Fourier deconvolution method of analysis, though the tether-in-a-cone model introduces constraints that can correct these errors. In the DEER experiment, the angular terms are of particular concern because of orientational selectivity. The bandwidths of the pulses are relatively narrow, so only a small subset of interacting spins are observed and may not represent the entire range of distances. At L-band, the angular terms in the dipolar interaction have no effect, because the g-anisotropy is negligible. Consequently, there is no asymmetric broadening or orientational selectivity, resulting in a true measurement of the average distance and distribution.

It is clear that L-band distance determination expands the range of distances that are measureable at physiological temperatures, but it must be noted that all the drawbacks of

CW distance determination remain. For example, CW calculations require two samples since deconvolution of the Pake requires the single and double labeled spectra. Therefore, the pros and cons of the pulsed and CW techniques must be weighed before opting for one or other. In many cases, it may be beneficial to both, particularly if the experimental conditions are suspected to alter the native structure.

5. Conclusion

L-band NARS EPR has been used to measure distances and distributions in the range of 15 to 30 Å accurately in two different systems. To ensure the maximum range of measureable distances and the most physiologically relevant measurement, experiments should be performed at room temperature and in the presence of the appropriate cosolvents that create an environment where the dipolar vector is not averaged out and the native structure is not altered. These conditions will vary with the size of the system under study. There remains room for improvement, as the range of measureable distances can be increased if narrower lines can be obtained. Such improvements present the possibility to observe large structural changes at physiologically relevant temperatures.

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- Inter-spin distances were measured using L-band NARS EPR in two systems.
- The upper limit of the CW distance determination technique was increased to 26 Å.
- VLF measurements were independent of angular terms in the dipolar function.

Fig. 1. The intensities and peak-to-peak linewidths of first harmonic spectra resulting from the pseudomodulation of the T4L-68 pure absorption spectrum with a maximum signal intensity of one.

Fig. 2.
The pure absorption spectrum of PA-3 and the resulting dipolar broadened spectra when a second spin is introduced. Spectra are normalized to spin concentration.

Fig. 3.
Pure absorption spectra of T4L mutants normalized to spin concentration.

Fig. 4. Simulations of dipolar broadened PA-3 pure absorption spectra with varying inter-spin distances and distribution widths. Inter-spin distances of 17.5 Å (top left), 20.0 Å (top right), 22.5 Å (bottom left), and 25.0 Å (bottom right) are presented with distribution widths of (a) 7 Å, (b) 5 Å, (c) 3 Å, and (d) 1 Å. Residuals between (A) d-c, (B) c-b, and (C) b-a are displayed below.

Table 1

Experimentally measured values as determined by L-band NARS EPR compared to the expected values calculated from a helical model¹⁰

	L-band r	L-band σ	Model r	Model σ
PA-3,11	15.6 Å	3.4 Å	17.0 Å	4.3 Å
PA-3,12	22.4 Å	3.8 Å	22.2 Å	2.9 Å
PA-3,13	20.4 Å	>4.0 Å	20.3 Å	5.3 Å
PA-3,14	18.9 Å	>3.5 Å	18.9 Å	4.8 Å
PA-3,18	25.9 Å	5.9 Å	26.4 Å	5.5 Å

Table 2

Experimentally measured values of T4L double mutants as determined by L-band NARS EPR compared to the values experimentally determined by DEER

	L-band r	L-band σ	DEER r	DEER σ
T4L-86,115	14.0 Å	2.8 Å	15.7 Å	0.5 Å
T4L-68,89	20.2 Å	2.6 Å	22.0 Å	1.8 Å
T4L-68,109	31.1 Å	2.1 Å	31.3 Å	0.9 Å