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Nanometer-Range Distance Measurement in a Protein Using Mn^{2+} Tags

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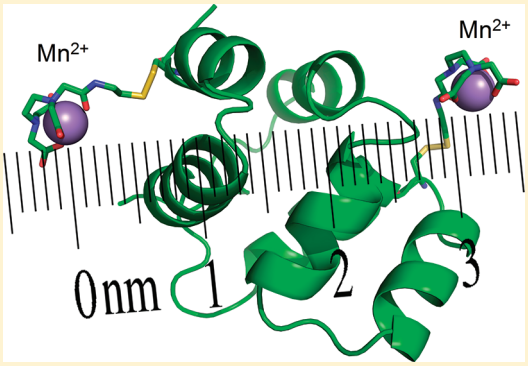
Supporting Information

ABSTRACT: Pulse electron paramagnetic resonance measurements of long-range (nm scale) distances between spin labels site-specifically attached to biomacromolecules have proven highly effective in structural studies. The most commonly used spin labels are stable nitroxide radicals, and measurements are usually carried out at X-band frequencies (~9.5 GHz, 0.35 T). Higher magnetic fields open new possibilities for distance measurements with increased sensitivity using alternative spin labels containing half-integer high-spin metal ions. Here we demonstrate W-band (95 GHz) pulse double electron−electron resonance (DEER) distance measurements in a protein labeled with two Mn^{2+}-EDTA tags. The distance distribution obtained is in excellent agreement with model calculations based on the known solution NMR structure. Thus, site-specific labeling with Mn^{2+} tags opens a highly promising approach to nanometer distance measurements in biological macromolecules.



SECTION: Biophysical Chemistry

Long-range (nm scale) distance measurements between specific sites in biological macromolecules offer important insight into their structure and interactions. In the past decade, such distance measurements by pulse electron paramagnetic resonance (EPR) techniques, often referred to as pulse dipolar spectroscopy (PDS), have proven highly efficient for nitroxide-labeled proteins and nucleic acids.[1−3] Distances in the range of 2−5 nm can be routinely accessed, and distances up to 8 nm can be determined under favorable conditions.[4,5] In proteins, site-directed spin labeling is usually achieved by covalent attachment of nitroxide compounds to cysteine residues that are either native or, more commonly, have been introduced at strategically chosen positions by site-directed mutagenesis.[6] The distance between the unpaired electrons of the nitroxides is determined by measurement of their dipolar interaction, ω_{dd}, following the relation

$$\omega_{dd} = \frac{\mu_0 \beta_e^2 g^2}{4\pi\hbar r^3}(3\cos^2\theta - 1) = \omega_{dd}^0(3\cos^2\theta - 1) \quad (1)$$

where r is the electron−electron distance and θ is the angle between the interelectron vector $\boldsymbol{r}$ and the external magnetic field. The measurements are carried out at low temperatures on frozen solutions.

The most popular PDS experiment is the four-pulse double electron−electron resonance (DEER) sequence,[7] often also referred to as PELDOR (pulse electron double resonance). DEER carried out at standard X-band frequencies (~9.5 GHz, 0.35 T) has become well-established. An order of magnitude increase in sensitivity can be gained by high-frequency/high-field DEER, provided that the microwave (mw) power is sufficient to generate short enough microwave pulses, as recently demonstrated at the W-band (95 GHz, ~3.5 T).[8,9] In addition, high-field measurements open new opportunities for the use of metal ions as spin labels. The EPR spectra of high-spin transition-metal ions with half-filled valence orbitals, such as Gd^{3+} ($S = 7/2$) and Mn^{2+} ($S = 5/2$), become much simpler at high fields, displaying an intense and relatively narrow central $|-1/2\rangle \rightarrow |1/2\rangle$ transition.[10,11] The width of this transition is proportional to D^2/ν_0, where D is the zero-field splitting (ZFS) parameter and ν_0 is the spectrometer frequency, leading to increased sensitivity with increased frequency. Capitalizing on this property, along with the short spin−lattice (T_1) relaxation times of metal ions and the efficient mw power utilization associated with high transition probabilities, a new type of spin label based on Gd^{3+} chelates has been introduced.[12−16] The new approach has been demonstrated for distance measurements of up to 6 nm in proteins and DNA molecules at Ka- (~32 MHz) and W-band frequencies.[15,16] At the W-band, sample quantities as little as ~0.1 nmol proved to be sufficient. Site-specific attachment of Gd^{3+} to proteins was achieved via metal-chelating tags bonded to cysteines, following the

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approach developed for lanthanide tags in paramagnetic NMR.[17]

The Gd^{3+} spin labels offer a decisive advantage over nitroxides in W-band distance measurements as they allow straightforward determination of the distance distribution[14] using the established DeerAnalysis software.[18] This advantage stems primarily from the isotropic character of the central transition and the broad distribution of ZFS parameters, which result in a featureless broad spectral line shape for the other transitions.[19] In contrast, the high-field EPR spectrum of nitroxides is governed by the resolved *g*-anisotropy. The resulting orientation selection complicates data analysis when the two nitroxides are motionally constrained, requiring the determination of five Euler angles in addition to the electron–electron distance to fit the data.[13,20,21]

Here we introduce a new family of spin labels based on Mn^{2+} chelates for long-range distance measurements in proteins at high magnetic fields. Mn^{2+} shares many of the spectroscopic advantages of Gd^{3+} and, by virtue of its different coordination chemistry and lesser toxicity in living systems, significantly broadens the scope of suitable metal tags. In particular, the smaller coordination number of Mn^{2+} compared to Gd^{3+} (6 versus 8–9) lends itself to the design of smaller tags.

We demonstrate the concept of Mn^{2+} labeling using the death domain of the p75 neurotrophin receptor (p75DD; residues 334–425). The solution NMR structure of p75DD is known.[22] The domain contains two cysteine residues, C379 and C416, which were conjugated with the ethylenediaminetetraacetic acid (EDTA) tag shown in Figure 1a. EDTA has a

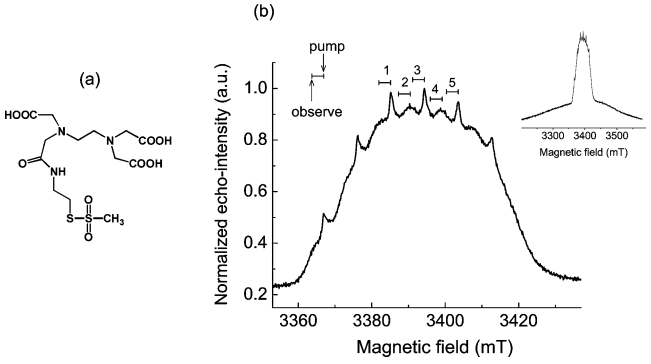

Figure 1. EDTA tag used (a) and W-band two-pulse echo-detected EPR spectrum of a 0.1 mM frozen solution of Mn^{2+}-EDTA labeled p75DD in a buffer of 20 mM HEPES, pD 7.0, in 80% D_2O/20% glycerol-d_8 at 10 K (b). The positions of the pump and observe pulses in the DEER experiments are indicated. The inset in (b) shows a wider range of the EPR spectrum.

very high affinity for Mn^{2+} at neutral pH ($\sim 10^{-11}$ M).[23] The tagging yield was nearly 100%. Details of the sample preparation are given in the Supporting Information. The W-band echo-detected EPR spectrum is shown in Figure 1b. The central transition is broad with barely resolved ^{55}Mn hyperfine splittings arising from a large ZFS parameter *D* of about 3000 MHz.[24] The six sharp low-intensity lines superimposed on the broad central line were attributed to a negligible amount ($\sim$1.6%) of noncoordinated Mn^{2+}.

We carried out four-pulse DEER measurements[7] (Figure S1, Supporting Information) at five different magnetic field positions within the central transition, as indicated in Figure 1b. The first modulation is clearly visible in all raw DEER data

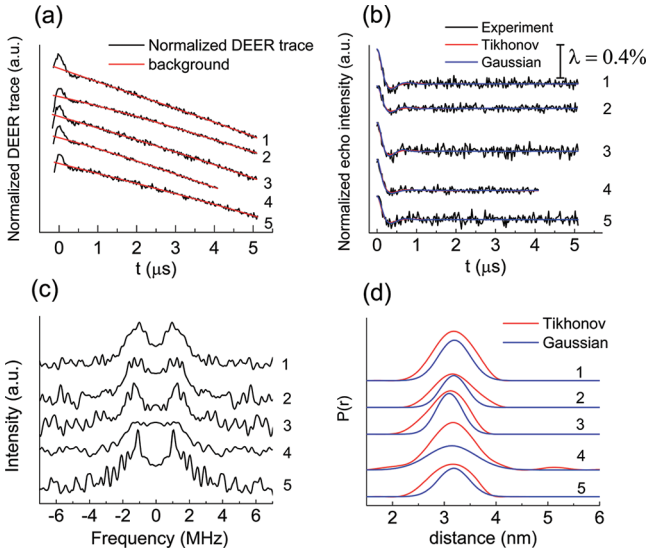

Figure 2. DEER data of Mn^{2+}-EDTA-labeled p75DD at 10 K. (a) Raw data with a background fit, (b) data after background removal and the fitted data obtained with the distance distributions shown in (d), (c) corresponding FT spectra, and (d) the distance distribution extracted from (b). $P(r)$ is the probability of finding a particular distance. The five different traces were obtained at the magnetic fields marked in Figure 1b. Experimental conditions: 15 ns pump pulse, 30 and 60 ns observe pulses, frequency difference of $\Delta\nu$ = 75 MHz, and repetition time of 1 ms. The accumulation time per trace was about 12 h, except for trace 4, which was accumulated in about 7 h.

(Figure 2a). Following background removal (Figure 2b), the Fourier transform spectra (Figure 2c) show a clear doublet with an average splitting of about 1.2 MHz, which can be assigned to the perpendicular singularities of the dipolar Pake doublet, corresponding to ω_{dd} for $\theta = 90°$. Variations in the shape of the doublets are attributed to variations in the background subtraction and the presence of noise. The Mn^{2+}–Mn^{2+} distance distributions extracted using DeerAnalysis[18] with a Gaussian fit or Tikhonov regularization are presented in Figure 2d. All distance distributions are rather broad (0.7–1.0 nm at half height), with a maximum at 3.18 nm, except for trace 3, which is shifted to 3.1 nm. The similarity of all traces demonstrates the absence of orientation selection. We made an effort to collect the data with a long dipolar evolution time of 5 μs to clearly demonstrate the potential of the method for measuring also longer distances, up to 6 nm[2], within a reasonable accumulation time. Furthermore, the long time was needed for an unambiguous determination of the background decay. In principle, it would have been sufficient to collect the DEER traces up to only 2 μs, which would require a much shorter accumulation time for the same S/N ratio. From the phase memory time measurement (Figure S3, Supporting Information), we estimated that a 2.7-fold increase in the echo intensity could be gained by using echo times of 2.7 rather than 5.7 μs. In this case, a measurement time of 1.7 h rather than $\sim$12 h would have been sufficient for each DEER measurement.

The observed modulation depth, λ, is very low, about 0.4% for all five measured DEER traces (Figure 2b). This value is somewhat lower than the value expected for the pump pulse duration and the line shape of the central transition ($\sim$0.6%).[14] The deviation may partly be due to some overlap between the bandwidths of the pump and observe pulses. Another factor could be the contributions of forbidden EPR transitions to the

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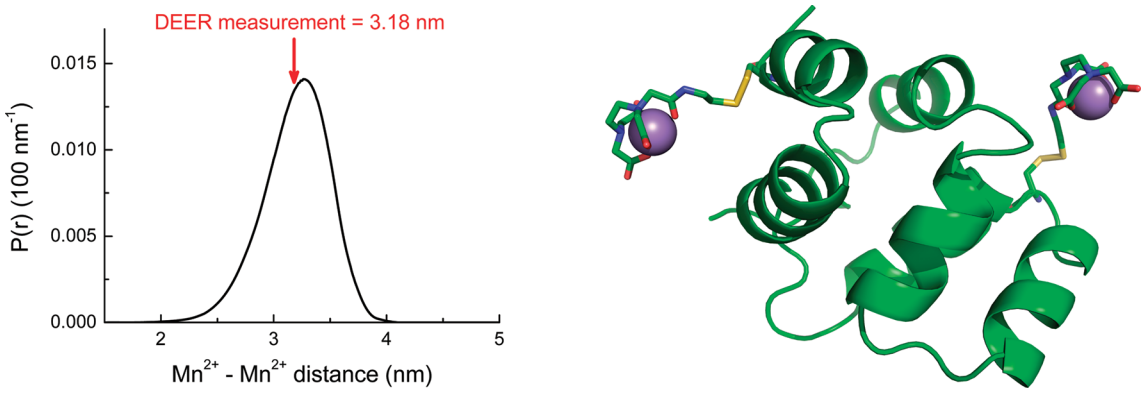

Figure 3. Modeled Mn^{2+}–Mn^{2+} distances. (Left) Calculated Mn^{2+}–Mn^{2+} distance distribution (for a bin size of 0.01 nm). The maximum is at 3.27 nm. The arrow indicates the value determined by DEER measurements. (Right) Ribbon drawing of a representative model of the structure of p75DD with Mn^{2+}-EDTA tags attached to C379 and C416.

spectrum due to the relatively large D, the behavior of which under DEER has not yet been studied. The sensitivity of the DEER experiment, S, is proportional to λV_0, where V_0 is the echo intensity at the observe frequency at the dipolar evolution time $t = 0$. As in the case of Gd^{3+} DEER measurements, the very low λ value is compensated for by a large V_0 and a short T_1.

To assess the accuracy of the distance measurement, we modeled the Mn^{2+}–Mn^{2+} distance by crafting two Mn^{2+}-EDTA tags onto the cysteine residues of the first conformer of the solution NMR structure of p75DD.[22] For each tag, 100000 models were generated by randomly varying the χ_2 angles of the cysteine residues as well as all rotatable bonds between the cysteine sulfur atoms and the Mn^{2+} chelate, which was modeled on the crystal structure of an Fe-EDTA-amide complex (Cambridge crystal database code QITHIK).[25] Models with van der Waals violations between the tags and the protein were removed. The resulting Mn^{2+}–Mn^{2+} distance distribution is shown in Figure 3. There is excellent agreement between the experimental and calculated distance distributions; the widths are comparable, and the maxima deviate by only about 0.1 nm.

p75DD differs from the full-length intracellular domain of the p75 neutrotophin receptor (p75ICD, residues 281–425) only by the absence of a highly mobile juxtamembrane polypeptide segment.[22] Earlier measurements of p75ICD labeled at the same positions with two dipicolinic acid Gd^{3+} tags gave a Gd^{3+}–Gd^{3+} distance distribution with a maximum at 2.9 nm,[13] that is, close to the Mn^{2+}–Mn^{2+} distance obtained here. The corresponding nitroxide–nitroxide distance was much shorter (2.5 nm), which must be attributed to nonuniform sampling of the conformational space of the tag, possibly arising from preferential hydrophobic interactions with the protein.[13]

For comparable D values, DEER measurements are expected to be more sensitive for Gd^{3+} than Mn^{2+} tags because the central line of the Mn^{2+} spectrum is split into six ^{55}Mn hyperfine lines, thus reducing both λ and V_0. This effect, however, is partly compensated for by the about 2-fold greater population difference of the central transition for Mn^{2+} in the temperature range of 6–10 K (Figure S2, Supporting Information). T_1 and the phase memory time, T_M, are major factors affecting the sensitivity of the DEER measurements.[1,2] The T_1 value of the Mn^{2+}-EDTA-tagged p75DD at 10 K was 150 μs, similar to that found for Gd^{3+}-labeled proteins (~100 μs),[13,26] and the T_M value was 6.8 μs (Figure S3, Supporting Information), that is, similar to the T_M of 7.6 μs measured for a Gd^{3+}-DOTA amide tag[27] in a nondeuterated protein.[16] Due to its large D value, the sensitivity of DEER experiments obtained with the Mn^{2+}-EDTA tag is less than what has been obtained with the Gd^{3+}-DOTA-amide tag.[16] This situation, however, may change completely if tags with low D values become available, in particular, in combination with a bimodal cavity[28] that allows placement of the pump and observe pulses on two neighboring narrow high-intensity hyperfine components (separated by ~280 MHz).

Beyond applications to proteins, our results open new possibilities for distance measurements in nucleic acids using a commercially available deoxythymidine-EDTA conjugate as a tag.[29] In addition, Mn^{2+} is often used as a paramagnetic substitute for Mg^{2+}, which is very abundant in biological systems.[30] Therefore, structural information can be derived from distance measurements between a Mn^{2+} ion substituting for Mg^{2+} and a site-specifically attached Mn^{2+}-EDTA tag.

In conclusion, W-band Mn^{2+}–Mn^{2+} distance measurements are attractive because of the $1/\nu_0$ dependence of the width of the central transition. On the basis of recent reports of Ka-band (~32 GHz) Gd^{3+}–Gd^{3+}[12,14,15] and Q-band (~34 GHz) Gd^{3+}–nitroxide[31] distance measurements, we expect that Mn^{2+}–Mn^{2+} distance measurements will be possible also at Q-band frequencies, albeit with lower sensitivity due to the increased line width and with limitation to relatively small D values. Large D values, as exhibited by Mn^{2+}-EDTA, would, in addition to low sensitivity, lead to complications in data analysis arising from contributions of nonsecular terms to the dipolar evolution.[12,14] The lower Q-band sensitivity, however, may be compensated for by more favorable relaxation times, shorter pulses, and a broader cavity bandwidth. Therefore, site-specific labeling with Mn^{2+} tags opens a highly promising approach to nanometer distance measurements in biological macromolecules at high magnetic field with sub nmol sensitivity, using commercially available tags. The different chemical nature of Mn^{2+} and Gd^{3+} ions greatly broadens the scope for the design of optimal tags for long-range distance measurements in different biomolecular systems.

■ ASSOCIATED CONTENT

Supporting Information

(1) Protocol for the preparation of the p75DD sample with Mn^{2+}-EDTA tags, (2) four-pulse double electron–electron resonance sequence, (3) Boltzmann population difference of

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the $|-1/2\rangle \rightarrow |1/2\rangle$ transition of Mn^{2+} and Gd^{3+} at different temperatures, and (4) W-band two-pulse echo decays and saturation recovery curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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