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Cysteine-Specific Cu^{2+} Chelating Tags Used as Paramagnetic Probes in Double Electron Electron Resonance

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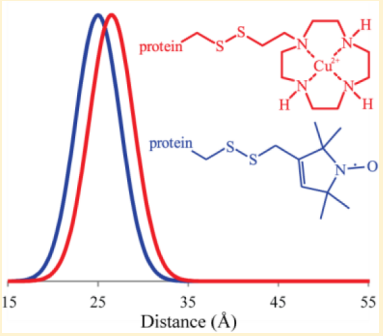
Supporting Information

ABSTRACT: Double electron electron resonance (DEER) is an attractive technique that is utilized for gaining insight into protein structure and dynamics via nanometer-scale distance measurements. The most commonly used paramagnetic tag in these measurements is a nitroxide spin label, R1. Here, we present the application of two types of high-affinity Cu^{2+} chelating tags, based on the EDTA and cyclen metal-binding motifs as alternative X-band DEER probes, using the B1 immunoglobulin-binding domain of protein G (GB1) as a model system. Both types of tags have been incorporated into a variety of protein secondary structure environments and exhibit high spectral sensitivity. In particular, the cyclen-based tag displays distance distributions with comparable distribution widths and most probable distances within 1−3 Å when compared to homologous R1 distributions. The results display the viability of the cyclen tag as an alternative to the R1 side chain for X-band DEER distance measurements in proteins.



INTRODUCTION

Double electron electron resonance (DEER) spectroscopy is an attractive electron spin resonance (ESR) technique that has allowed for the experimental measurement of distance distributions between multiple paramagnetic species in a variety of biological systems.[1−3] Paramagnetic species are typically not native to many protein systems and thus are introduced using a technique known as site-directed spin labeling (SDSL).[4−6] In SDSL, paramagnetic tags are commonly incorporated through direct attachment to cysteine residues which have been engineered into the protein at sites of interest via mutagenesis. By far, the most common paramagnetic tag is the methanothiosulfonate spin label, or MTSSL. MTSSL reacts specifically with the free thiol group of cysteine residues, and the result is the nitroxide side chain known as R1, as shown in Figure 1a. The use of R1 in DEER distance measurements as well as its various other applications have been reviewed extensively.[4−9]

In addition to stable organic radicals, an alternate source of ESR active species within proteins is paramagnetic metal ions. The simplest cases are those proteins that bind these paramagnetic metals naturally, and if a protein contains multiple metal centers, DEER can be utilized to elucidate structural and dynamical information. Additionally, SDSL can be used in conjunction with these native metal binding sites, and DEER can be performed between the metal center and the spin-labeled site(s). Applications of DEER measurements using natural metal binding sites has been reviewed recently.[10]

An alternate means of utilizing paramagnetic metal ions as DEER probes is through site-specific incorporation of tags that strongly chelate paramagnetic metals such as Gd^{3+}.[11] In addition to Gd^{3+} tags being able to take advantage of the increased sensitivity at high field,[12] these metal chelating tags have displayed distinct advantages over R1 in highly relevant biological environments. Within lipid membranes, certain Gd^{3+} tags have displayed less conformational bias due to the hydrophobic environment as compared to R1 and thus may provide a more representative distance measurement within the membrane.[13] Metal-based DEER measurements also appear to be less affected by multispin effects in proteins containing more than two spins.[14] Additionally, Gd^{3+} tags have displayed much greater stability to the reducing conditions of living cells as compared to R1 for *in-cell* ESR distance measurements.[15] Taken together, metal chelating tags are advantageous for measuring distances in some biological environments.

High-field Gd^{3+} DEER measurements have been performed at W band (~95 GHz) or in some cases at Ka band (~32 Ghz). While Gd^{3+}-R1 DEER measurements have been performed at the X band (~9.5 GHz) on a model system, the measurement suffered from low signal-to-noise[16] due to the broadening of the central adsorption in the Gd^{3+} spectrum. Given the prevalence of X-band instruments and the advantages these tags can offer, it is important to develop alternative metal chelating tags for use at X-band frequencies.

An additional group of metal chelating tags being developed for protein structural studies is those that strongly chelate Cu^{2+}, and indeed, such tags have been successfully utilized recently for measurement of electron−nucleus distance-dependent

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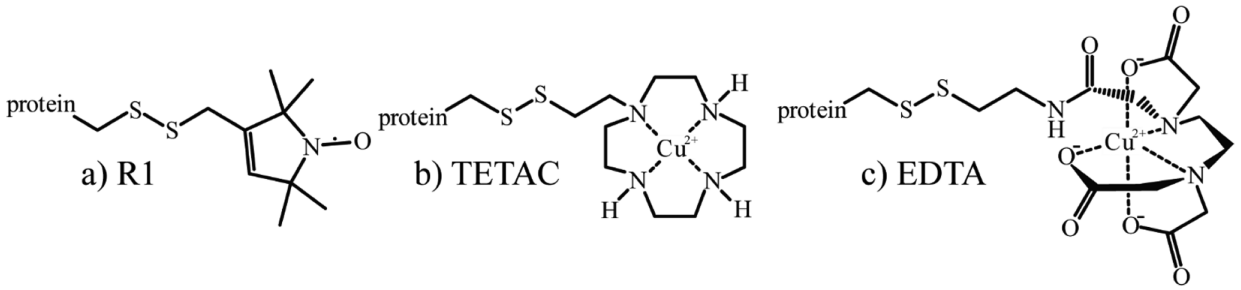

Figure 1. Three paramagnetic side chains used as DEER probes after attachment to a cysteine residue: (a) R1 is the common nitroxide side chain, while (b) TETAC and (c) EDTA are both Cu^{2+} chelating tags utilized here.

paramagnetic relaxation enhancements by solid state nuclear magnetic resonance (NMR) spectroscopy.[17−20] Similar to MTSSL and all Gd^{3+} tags, the tags utilized thus far react specifically with cysteine residues. While intrinsically bound Cu^{2+} ions have been used extensively for X-band DEER measurements in model systems and several proteins,[10] the use of Cu^{2+} chelating tags has not been explored in this context. Here, we utilize two such Cu^{2+} tags as X-band DEER probes and compare the results with the common R1 spin label. The tags selected are the 1-(2-(pyridin-2-yldisulfanyl)ethyl)-1,4,7,10-tetraazacyclododecane (TETAC) tag[20] and the commercially available ethylenediaminetriacetic acid (EDTA) tag,[21,22] the latter of which has been also utilized to chelate Mn^{2+} for use in DEER distance measurements.[11] The resultant side chains for the TETAC and EDTA tags are shown in Figure 1b and 1c. Note that these tags are ∼20% and ∼40% larger than the R1 side chain. All tags were incorporated into various double-cysteine mutants of the 56-residue B1 immunoglobulin binding domain of protein G (GB1), and the results presented here display the utility of these tags as X-band, paramagnetic metal probes for use in protein distance measurements.

EXPERIMENTAL METHODS

Construction of GB1Mutants. The plasmid encoding for wild-type GB1 was kindly provided by Prof. Angela Gronenborn (University of Pittsburgh). The cysteine mutation locations were chosen to represent a variety of solvent-exposed β-sheet locations (I6, N8, and E15) as well as a solvent-exposed α-helix location (K28). Mutations were performed one at a time, and mutant plasmid was used to create the desired double mutants (6/28, 8/28, and 15/28). Each mutant was created using the appropriate plasmid DNA, the primers encoding for the desired mutation (Invitrogen, Carlsbad, CA), and the KAPA Hifi Hotstart Ready Mix (Kapa Biosystems, Cape Town, South Africa). Resultant PCR reaction mixtures were treated with DpnI (New England Biolabs, Boston, MA), transformed into XL1-Blue Supercompetent cells (New England Biolabs), and grown overnight on culture plates containing Luria−Bertani (LB) broth with 100 mg/mL ampicillin. Colonies were picked and grown overnight in 50 mL of LB with 100 mg/mL ampicillin, and the plasmid DNA was purified using the PureYield Plasmid Midiprep System (Promega, Madison, WI). All mutations were confirmed by DNA sequencing (Genomic and Proteomics Core Laboratories, University of Pittsburgh, Pittsburgh, PA) and subsequently transformed into BL21(DE3) competent cells (New England Biolabs) for expression.

Protein Expression, Purification, and Labeling. Expression and purification of all GB1 mutants was performed as previously described.[23] The labels used were the TETAC tag (synthesis described previously),[20] the methanothiosulfonate spin label (MTSSL), or the [*S*-methanethiosulfonylcysteaminyl] ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid chelating tag (MTS-EDTA); the latter two tags were both purchased from Toronto Research Chemicals (Toronto, Canada). Labeling with MTSSL was performed as previously described.[23] Labeling with MTS-EDTA and TETAC were performed similarly except since both tags are water soluble no DMSO was utilized in the labeling process. Removal of the excess chelating tags was performed similar to the procedure for MTSSL.[23]

ESR Measurements. DEER distance measurements were performed on MTSSL-labeled mutants as previously described.[23] For the MTS−EDTA and TETAC-labeled samples, the samples were at a concentration of 0.5 mM in 50 mM *N*-ethyl morpholine (NEM) at pH 7.4, 25% glycerol, and 0.95 mM isotopic $^{65}CuCl_2$ (Cambridge Isotopes, Tewksbury, MA). The Cu^{2+} chelating samples were slightly underloaded with Cu^{2+} due to Cu^{2+} interacting with GB1 elsewhere and leading to unwanted signals.[24]

All DEER distance measurements were performed on a Bruker Elexsys 580 spectrometer equipped with a Bruker ER4118X-MD5 resonator or a Bruker ElexSys E680 X-band FT/CW spectrometer equipped with a Bruker EN4118X-MD4 resonator. The temperature for all experiments was controlled using an Oxford ITC503 temperature controller with an Oxford ER 4118CF gas flow cryostat. Distance measurements for both Cu^{2+} samples were performed at 20 K. The four-pulse sequence utilized for the measurements was $(\pi/2)\nu1-\tau1-(\pi)\nu1-T-(\pi)\nu2-\tau2-(\pi)\nu1-\tau2-$echo. For the distance measurements displayed in Figure 3, the pump pulse ($\nu2$) was placed at the maximum of the Cu^{2+} spectrum and the observer pulses ($\nu1$) are placed 150 MHz downfield from the maximum. The observer pulse ($\nu1$) lengths were 16 and 32 ns for the $\pi/2$ and π pulses, and the pump pulse ($\nu2$) length was 16 ns. The parameter τ_1 was set to 200 ns and T to 160 ns initially with T being increased by 10 ns for 128 steps. τ_2 was adjusted such that $\tau_2 + T = 1300$ ns. DEER data collection times for all spectra are comparable (∼20 h for all R1, EDTA−Cu^{2+}, and TETAC−Cu^{2+}-labeled samples).

RESULTS AND DISCUSSION

The Cu^{2+} binding tags and the common R1 spin label were attached to several double-cysteine mutants of GB1 as described below. The three-dimensional structure of GB1 is well known[25−28] consisting of a single α-helix and a four-stranded β-sheet (Figure 2). For this comparison, three different double-cysteine mutants were utilized, each containing the same solvent-exposed α-helical site (K28C) and a single solvent-exposed β-sheet site. The three β-sheet sites were

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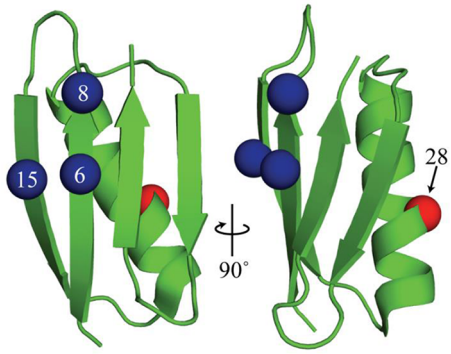

Figure 2. NMR structure of GB1 (PDB ID 2LGI)[28] showing all labeled sites. Each DEER pair included the α-helix site 28 and one of the β-sheet sites, either 6, 8, or 15.

selected to represent a variety of β-sheet locations including an internal strand site (I6C), a previously investigated internal strand site neighboring a β-turn (N8C),[23] and an external strand site (E15C). The relative location of all labeled sites can be seen in Figure 2. For all three GB1 double mutants (6/28, 8/28, and 15/28), each was tagged with the three different tags (R1, TETAC–Cu^{2+}, and EDTA–Cu^{2+}) and X-band DEER measurements were performed.

Figure 3a, 3b, and 3c shows the baseline-subtracted Cu^{2+} signal for the 6/28, 8/28, and 15/28 mutants, respectively. The raw signals of the R1 measurements are provided in Figure S3, Supporting Information. Comparatively, the time domain signals for the Cu^{2+}-tagged mutants mostly display modulation depths of up to 10%, which is sufficient to achieve a quality signal-to-noise at X band. A lower modulation depth was achieved in 8/28 TETAC–Cu^{2+}, which is likely due to incomplete labeling (estimated to be ~74% from the modulation depth).[29] Nevertheless, reasonable signal-to-noise was achieved for this mutant. For Gd^{3+} DEER measurements, the commonly low modulation depths of ~1% have been attributed to the presence of free Gd^{3+} ions masking the DEER effect.[12] Cu^{2+}–DEER offers a significant advantage in that free Cu^{2+} is EPR silent[30] in the *N*-ethylmorpholine (NEM) buffer at pH 7.4, which was used in these measurements. Thus, with NEM buffer, the presence of free ions is not a concern. Both tags were also probed for the presence of orientational selectivity by collecting the DEER spectrum at multiple magnetic fields (cf. Figures S1–S3, Supporting Information). Very weak effects of orientational selectivity were observed only for the TETAC tag. Fits to the data obtained at different magnetic fields indicate the distribution width is similar, but the most probable distance varies only by ~1 Å. Thus, orientational effects are largely reduced possibly due to an orientational distribution between the two Cu^{2+} centers.[10,31] Accordingly, the remaining samples were collected at a single magnetic field and analyzed using DEERAnalysis.[32] Given that single distances were expected for all measurements and fitting with model distributions is known to improve the reliability of the analysis, all data were subsequently fit with single-Gaussian distributions.[32]

The resultant distance distributions from all measurements are shown in the Figure 3d, 3e, and 3f. For each of the three mutants, the EDTA–Cu^{2+} tag shows the longest most probable distance when compared to the other two tags. These longer distances are expected given the relative structures of the tags, as shown in Figure 1. R1 has five bonds between the protein backbone and the nitroxide ring, TETAC has six between the

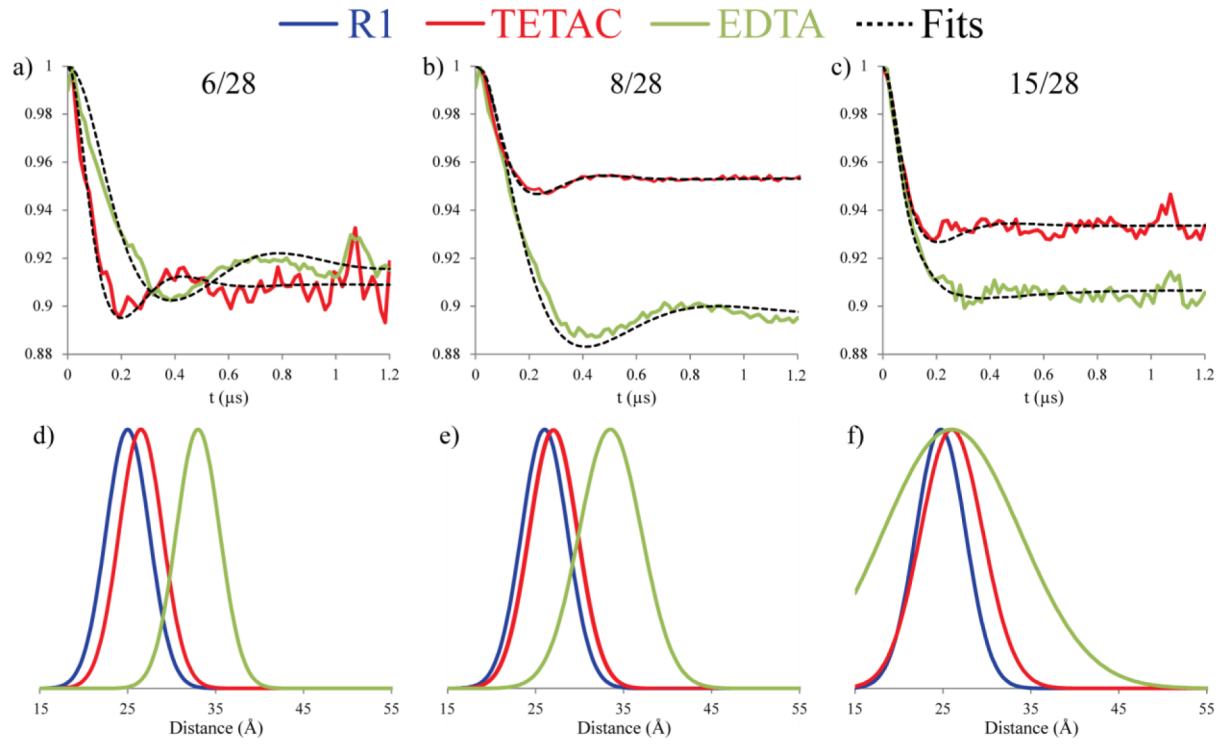

Figure 3. Baseline-subtracted time domain DEER data for the double-cysteine GB1 mutants (a) 6/28, (b) 8/28, and (c) 15/28 tagged with TETAC–Cu^{2+} (red) or EDTA–Cu^{2+} (green) and the best single-Gaussian fits (dotted black) using DEER analysis (due to differences in scale, the R1 time domain data is shown separately in Supporting Information). Bottom panels show the resultant distance distributions for (d) 6/28, (e) 8/28, and (f) 15/28 tagged with R1 (blue), TETAC–Cu^{2+} (red), and EDTA–Cu^{2+} (green).

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backbone and the chelating motif, while EDTA has nine. Consequently, the resultant TETAC−Cu^{2+} DEER distance distributions show remarkable similarity with each of the R1 distributions (Figure 3). For the various mutants, the most probable distance for the TETAC−Cu^{2+} distributions, as compared to the R1 distributions, only differ by 3 Å for the 6/28 mutant, 1 Å for the 8/28 mutant, and 1 Å for the 15/28 mutant. Additionally, the breadth of the distributions are comparable to the R1 distributions for all cases. Despite the variety of solvent-exposed protein environments probed here, the corresponding DEER distributions match well, suggesting that the TETAC−Cu^{2+} side chain is a reasonable alternative to the widely utilized R1 spin label for use in DEER distance measurements.

In summary, this work displays the utility of Cu^{2+} chelating tags as X-band DEER spin probes in various solvent-exposed protein environments. Both Cu^{2+} chelating tags used here exhibit minimal orientational selectivity at X-band frequencies. Additionally, the tags display sufficient sensitivity, partly due to the use of NEM buffer, which eliminates the potential negative effects caused by the presence of excess Cu^{2+} ions. The TETAC−Cu^{2+} tag is closest to R1 in terms of the measured distance distribution as compared to other transition metal chelating tags utilized thus far, making it a viable alternative spin label for use as an X-band DEER probe. While the environments probed here are all solvent-exposed sites, it will be interesting to determine if these Cu^{2+} tags offer distinct advantages in the membrane environment given the structural similarity of the Gd^{3+} and Cu^{2+} chelating tags. Additionally, the possible use of these Cu^{2+} tags as in vivo DEER probes is an exciting prospect that may allow for in-cell measurements to be made with the more commonly utilized X-band instruments.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods describing expression and purification of GB1 mutants; DEER data collection; figures containing the time domain and resultant Pake patterns for the DEER measurements made at various magnetic field strengths for 8/28 tagged with TETAC−Cu^{2+} and EDTA−Cu^{2+}, and the baseline-subtracted time domain data for the R1 measurements with single-Gaussian fits. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DEER, double electron electron resonance; ESR, electron spin resonance; SDSL, site-directed spin labeling; MTSSL, methanthiosulfonate spin label; TETAC, 1-(2-(pyridin-2-yldisulfanyl)ethyl)-1,4,7,10-tetraazacyclododecane; EDTA, ethylenediaminetriacetic acid; GB1, B1 immunoglobulin binding domain of protein G; NEM, *N*-ethylmorpholine; LB, Luria−Bertani

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