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Letter

dHis-troying Barriers: Deuteration Provides a Pathway to Increase Sensitivity and Accessible Distances for Cu^{2+} Labels

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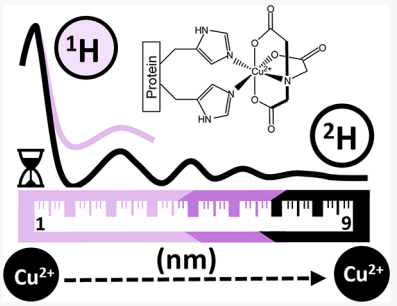
ABSTRACT: Recently, site-directed Cu^{2+} labeling has emerged as an incisive biophysical tool to directly report on distance constraints that pertain to the structure, conformational transitions, and dynamics of proteins and nucleic acids. However, short phase memory times inherent to the Cu^{2+} labels limit measurable distances to 4−5 nm. In this work we systematically examine different methods to dampen electron−nuclear and electron−electron coupled interactions to decrease rapid relaxation. We show that using Cu^{2+} spin concentrations up to ca. 800 μM has an invariant effect on relaxation and that increasing the cryoprotectant concentration reduces contributions of solvent protons to relaxation. On the other hand, the deuteration of protein and solvent dramatically increases the duration of the dipolar modulated signal by over 6-fold to 32 μs. Based on this increase in signal longevity, distances up to 9 nm and beyond can potentially be measured with Cu^{2+} labels.



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The ability to measure long-range distance distributions has given pulsed electron paramagnetic resonance (EPR) a powerful role in the elucidation of the atomic-scale details of protein structure and function.[1−5] By introducing two or more EPR-active species into a macromolecule via spin labeling,[6] pulsed EPR methods can determine interspin distance distributions by measurement of the dipolar interaction.[7,8] Such distance distributions are exploited to shed light on biomolecular assembly,[9,10] quaternary structure,[11] induced conformational changes,[12,13] and substrate/metal binding sites.[14,15]

Recently, a straightforward site-directed labeling technique through Cu^{2+} coordination to strategically substituted double histidine sites (dHis) into a protein backbone has been developed.[16] Specifically, histidine residues are placed at *i,i*+4 positions for α-helices and *i,i*+2 positions for β-sheets to enable specific cis coordination of a Cu^{2+}-NTA (nitrilotriacetate) complex.[17] Such site-directed Cu^{2+} labeling creates a small rigid spin label with a restricted spatial occupancy, providing distance distributions up to 5 times narrower than common nitroxide labels.[18] In return, the rigidity allows for the measurement of distance distributions that are more directly related to conformational changes,[19,20] site-specific dynamics,[21] and relative orientations of the protein.[22] The labeling is implementable in a variety of buffers,[23] the Cu^{2+}-NTA chelation to dHis has sufficient affinity, especially at cryogenic temperatures,[24,25] and is relatively resistant to competition by other metal ions.[26] Additionally, the combination of dHis-based Cu^{2+} labeling with nitroxides can provide a pathway to measurements at 100 nM protein concentration.[27] Following these results, simple Cu^{2+} labeling strategies, such as using a 2,2′-dipicolylamine (DPA) Cu^{2+} chelator, have also been developed for nucleic acids to report accurately on duplex backbone distances.[28]

Despite these early promising results, the phase memory relaxation time (T_m) of the Cu^{2+} labels at 20 K has been found to be ca. 3.5−4.0 μs in a wide variety of systems.[29] Such short values of T_m lead to echo signals of low intensities, which places a practical limit on the upper distances. With current technology, the measurable modulated signal between Cu^{2+} spin labels only lasts ca. 4−5 μs. In this work we show the potential of deuteration to significantly go beyond the current bottleneck that relaxation times place on distance measurements using Cu^{2+} spin labels.

For this work, the E15H/T17H/K28H/Q32H mutant of protein GB1 was labeled by the addition of a 1:1 molar equivalent of Cu^{2+}-NTA per dHis site in 50 mM MOPS buffer at a pH of 7.4. All stock preparation and protein expression details are presented in the Supporting Information (SI). First, we used a two-pulse sequence to measure the T_m at different protein concentrations, temperature, and the amount of cryoprotectant. These factors affect the modulation of electron−nuclear dipolar coupling and nuclear spin diffusion, which are leading contributors to T_m[30−33] at cryogenic temperatures. The data, shown in the SI, suggests that the T_m is largely invariant to Cu^{2+} spin concentration in the range of 20−800 μM (cf. Figure S1A). In addition, the balance of T_m

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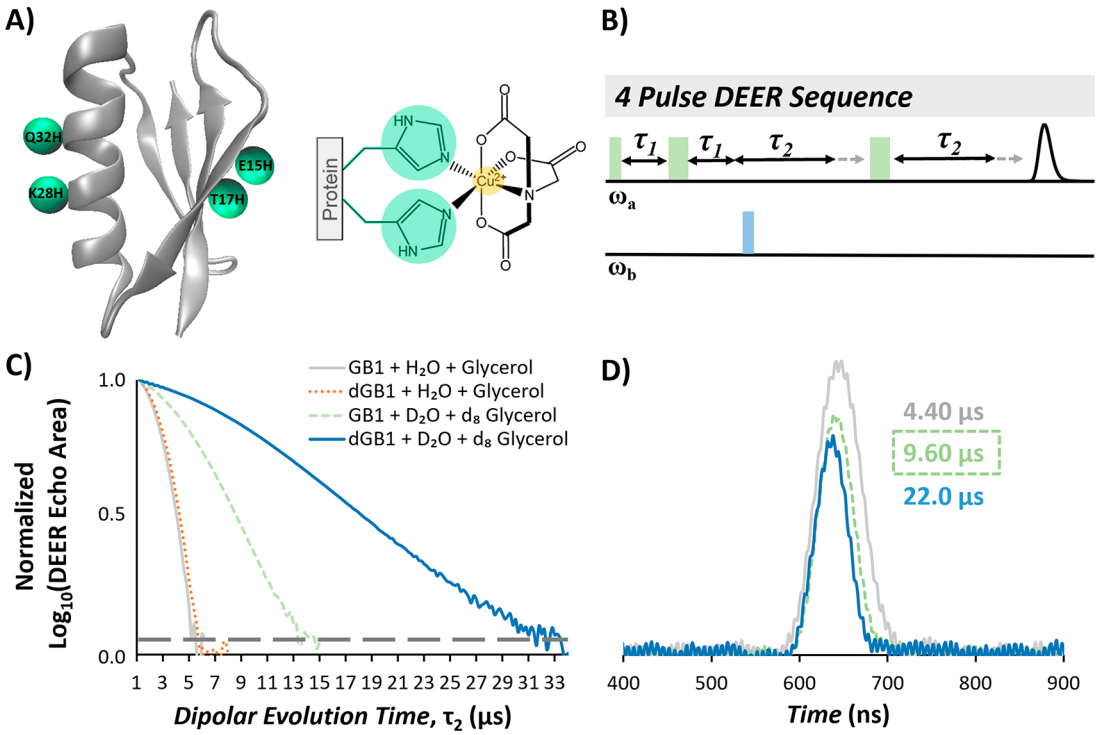

Figure 1. (A) Crystal structure of GB1 (PDB: 4WH4) showing the positions of the dHis sites. Green spheres represent His residues. The chemical structure of the dHis-Cu^{2+}-NTA complex is shown on the right. (B) The four-pulse DEER sequence utilized in the echo tracking experiment. (C) The integrated stimulated DEER echo as a function the dipolar evolution time (τ_2) is shown for each sample. Each echo was one scan at 20 shots per point. All other parameters were held constant and are described in the SI. The echo areas are presented as normalized logs, and the dashed gray line represents the point when the echo falls beneath the noise. Deuteration of protein and solvent leads to a dramatic increase in echo size at higher dipolar evolution times. (D) Illustrative examples of stimulated DEER echoes for each sample combination. The echo of the fully deuterated system is comparable to that of the non-deuterated sample (with τ_2 = 4.40 μs) even at a much larger dipolar modulation time of 22.0 μs.

and T_1 suggests that 18 K is a reasonable operating temperature to optimize T_m without increasing collection time (cf. Figure S1A). The glycerol concentration has a more pronounced effect on the T_m, and a ratio of 40% (v/v) glycerol maximizes the T_m (cf. Figure S1B). Based on these results, we determined that 150 μM Cu^{2+}-NTA, 40% glycerol (v/v), at an operating temperature of 18 ± 1 K optimizes the T_m to 4.1 μs. These optimized conditions were used for all samples presented throughout this work.

Next, we investigated the effects of deuteration on the T_m of Cu^{2+} spin labels, given that previous work on nitroxide spin label systems has demonstrated that deuteration of solvent protons, biomolecules, and cryoprotectants provides dramatic increases in T_m[34,35] by reducing the electron−nuclear dipolar interaction. Figure 1C shows the integrated double-electron electron resonance (DEER)[36] echo as the dipolar evolution time, τ_2, is increased sequentially by 0.2 μs from an initial value of 1.2 μs. In DEER, the longer the distance, the longer the duration of τ_2 that is required for experimentation (Figure 1B). Interestingly, the use of D_2O and d_8-glycerol led to significant gains in the echo intensity at large dipolar evolution times and, indeed, sustaining a measurable signal for ca. 13 μs.

Next, we sought to increase the relaxation time even longer by deuterating GB1. Accordingly, we deuterated GB1 using a cell expression protocol described in the SI.[37] Deuteration of GB1 was estimated to be at least 76% isotopically labeled, as determined by liquid chromatography−mass spectrometry (Figure S2). In addition, continuous-wave (CW) EPR spectra showed the same coordination of Cu^{2+}-NTA to both the deuterated and naturally abundant GB1 dHis mutants (Figure S3). Interestingly, when deuterated dGB1 was used with non-deuterated water and glycerol, there was only a small gain in the phase memory time, leading to marginal improvements in the upper range of dipolar evolution time (Figure 1C). This result is likely due to dGB1 amide deuterium−proton exchange during the 30 min incubation time for coordination of Cu^{2+}-NTA to dHis prior to freezing.[23] The hydrogen−deuteration exchange halftimes for solvent-exposed amide protons have been shown to occur in seconds at a physiological pH.[38] Additionally, solvent atoms are densely packed around the spin center, which provides a significant contribution to signal dephasing.[39]

To overcome relaxation induced by solvent protons, we incorporated combinations of d_8-glycerol and D_2O with dGB1. Protein deuteration leads to a substantial increase in the size of the echo at large dipolar evolution times, with the signal sustaining until 32 μs (cf. Figure 1C). These results are reasonable, since the close proximity of the Cu^{2+} label to the protein backbone and side chains is expected to contribute most efficiently to relaxation.[40] Also, this data demonstrates the necessity of combining deuterated protein with deuterated solvent to drive long relaxation times. In non-deuterated systems, a dipolar evolution time of 4.4 μs is generally the upper limit for such Cu^{2+} labels, which imposes a practical limit of ca. 4−5 nm on the range of measurable distances. Figure 1D shows that, with complete deuteration, the echo at 22 μs is comparable to the 4.4 μs echo, demonstrating the enhancement in feasible values of dipolar evolution times. Indeed, with deuteration, longer dipolar modulation times up to ca. 32 μs become possible (Figure 1C). Such a striking amplification of

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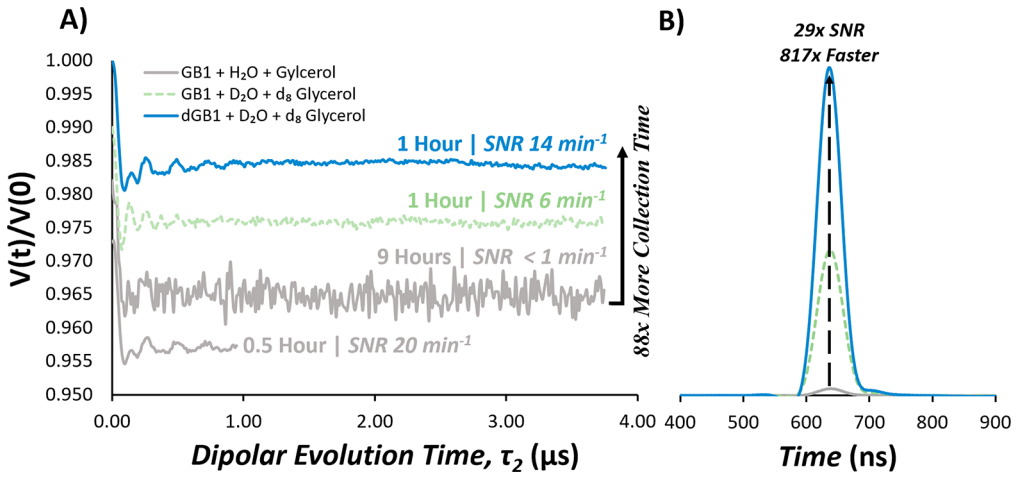

Figure 2. (A) Background-subtracted DEER time traces for the different sample deuteration combinations. Each DEER was collected using a 100 MHz observer-pump offset, with the pump pulse applied at the maximum magnetic field. All other parameters were held constant for each sample and are provided in the SI. A 1 μs DEER time trace for a naturally abundant GB1 system is also shown for comparison. (B) Refocused DEER echoes and estimated experiment run time comparisons for each sample shown at a 4 μs.

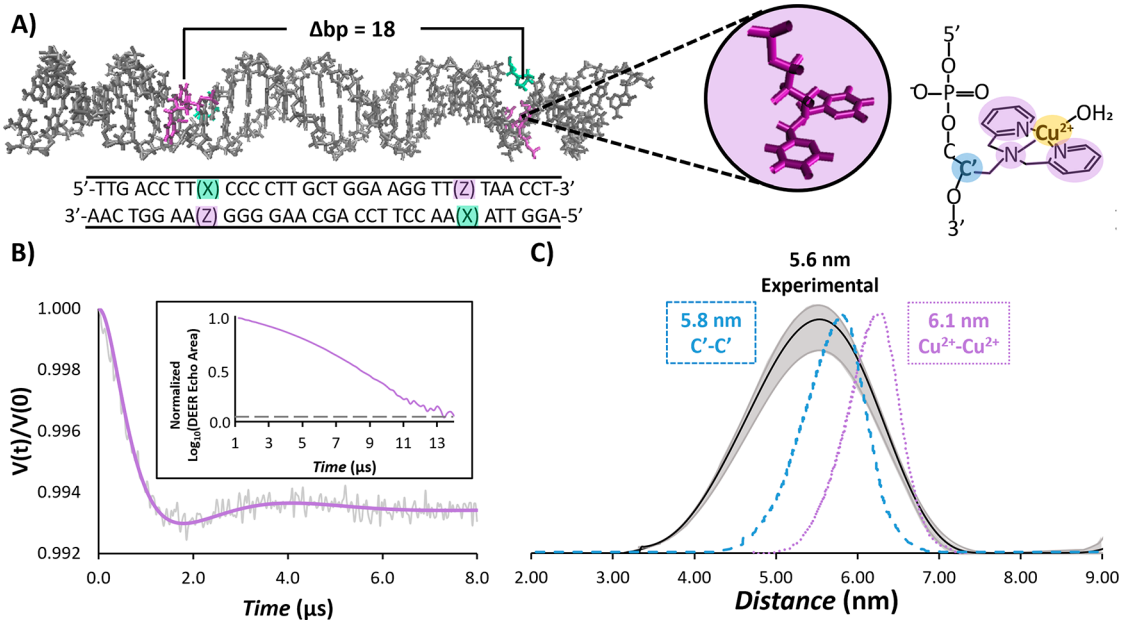

Figure 3. (A) Structure of the DPA-DNA + Cu^{2+} duplex with a base-pair separation of 18 between DPA chelators. X represents the substituted DPA, and Z is the complementary abasic site. The structure of DPA phosphoramidite + Cu^{2+} is shown on the right. (B) Background-subtracted DEER time trace of a DPA-DNA + Cu^{2+} duplex and simulated fit (purple). The inset depicts the normalized log of the integrated DEER stimulated echo versus the dipolar evolution time.tracking at (C) Extracted distance distribution centered at 5.6 ± 0.7 nm. The 2.0 μs MD showed a C′−C′ distance of 5.8 ± 0.39 nm (dashed blue) and a Cu^{2+}−Cu^{2+} distance of 6.1 ± 0.29 nm (dotted purple).

dipolar evolution time offers a pathway for Cu^{2+} spin labels to measure distances up to 9 nm and beyond. Further improvements can be made by the use of deuterated buffer, if available, and by exploiting Carr−Purcell pulse sequences to increase relaxation times.[41] Specifically, such sequences increase signal duration by 92, 44, and 9.8% for GB1 + H_2O + glycerol, GB1 + D_2O + d_8-glycerol, and dGB1 + D_2O + d_8-glycerol, respectively (cf. Figure S4).

To exemplify the effects deuteration has on DEER sensitivity with respect to Cu^{2+} spin labels, time traces were collected for the various GB1 samples shown in Figure 2. The E15H/T17H/K28H/Q32H GB1 mutant has a most probable distance of 2.3 nm[23] and requires ∼1 μs of dipolar evolution time to accurately capture multiple periods of modulation. However, to replicate long-range distance measurements, DEER time traces were also collected using a 4 μs acquisition time with 398 points at a 10 ns step size (Figure S6). An acquisition time of 4 μs was chosen as this dipolar evolution time is near the upper limit on non-deuterated samples. Figure 2A shows the dramatic gain in sensitivity for long-range DEER distance measurements through system deuteration. With deuteration, a 4 μs time trace can be collected in only one hour with a signal-to-noise ratio (SNR) on par with that of a 1 μs DEER on the non-deuterated sample. Specifically, system deuteration leads to a ca. 29-fold improvement in the SNR of the refocused DEER echo (cf. Figure 2B), which expedites collection time extraordinarily (Figure 2B). In fact, substantial gains are achieved simply by using D_2O or d_8-glycerol with

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naturally abundant biomolecules. We did not analyze these data to extract distance because an acquisition at one field with dHis at Q-band is susceptible to effects of orientation selectivity.[22] Nevertheless, the data shows the clear enhancement in sensitivity by deuteration.

Next, we performed DEER on a DPA-DNA + Cu^{2+} duplex with an 18 base-pair separation (Figure 3A) to observe the impact of using only d_8-glycerol and D_2O on long-range distance measurements for Cu^{2+} spin labels (Figure 3). The inset to Figure 3A shows that the DEER echo decay as a function of the dipolar evolution time was consistent with the D_2O + d_8-glycerol echo decay for GB1 in Figure 1C. Subsequently, a DEER signal with 8 μs of dipolar evolution time was collected using Cu^{2+} spin labels (Figure 3B, Figure S7). The extracted distance distribution gave a most probable distance of 5.6 ± 0.7 nm and a distribution breadth characteristic of the DPA motif[42] (Figure 3C). The experimental most probable distance is consistent with the prediction of C′−C′ and Cu^{2+}−Cu^{2+} distances from a 2.0 μs Molecular Dynamics (MD) simulation. Note that previous work has shown that a MD trajectory of such a length is sufficient to reasonably estimate the most probable distance but may not be sufficient to capture the full distribution width due to slow mobility of the DPA side chains.[43]

In summary, we present a systematic examination of factors that enhance the phase memory times for Cu^{2+} labels. In particular, we show that deuteration of the solvent and cryoprotectant can provide improvements that allow for the rapid measurement of distances up to 6.7 nm. For instance, we applied only D_2O and d_8-glycerol to efficiently obtain DEER distances with an 8 μs dipolar evolution time. In addition, deuteration of the biomolecule can readily extend the measurable distances using Cu^{2+} labels to 9 nm and beyond by dramatically increasing spin phase memory relaxation. Further improvements can be made by enhancing the modulation depths in DEER by using specialized shaped pulses.[44] Cumulatively, this work offers a straightforward sample preparation scheme to increase sensitivity, reduce experiment run times, and increase the ceiling on accessible distance constraints for Cu^{2+} labels with uncomplicated deuteration schemes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.1c01002.

Experimental methods for deuterated and naturally abundant GB1 expression and purification, Cu^{2+}-NTA stock preparation, and GB1 and DNA-DPA EPR sample preparation; continuous-wave and pulsed EPR instrumentation and parameters; complementary data for operating temperature, glycerol concentration, and spin concentration phase memory time and spin−lattice relaxation trends; GB1 continuous-wave spectra; and raw DEER time traces (PDF)

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Notes

The authors declare no competing financial interest.

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