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Pulsed ELDOR Determination of the Intramolecular Distance between the Metal Binding Sites in Dicupric Human Serum Transferrin and Lactoferrin

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Serum transferrin (Tf) and lactoferrin (Lf) are members of an important group of iron-binding and transport proteins.[1,2] A single polypeptide folds into two lobes of similar structure, each binding a single Fe^{3+} ion.[3−5] The iron can be removed and replaced by a number of other metal ions, while retaining the overall protein structure.[6−9] Of great interest is how these proteins interact with their bacterial[10] and mammalian[11] receptors and how changes to the tertiary structure upon binding ultimately lead to iron release.[12]

Pulsed electron−electron double resonance (ELDOR) is a technique which allows the estimation of molecular distances from the dipolar interaction, ν_{DD}, between paramagnetic centers.[13−15] This is given by eq 1:

$$\nu_{DD}(\theta,r) = \frac{g_1 g_2 \mu_0 \mu_B^{2}}{4\pi h}\frac{1}{r^3}(3\cos^2\theta - 1) \tag{1}$$

where g_1 and g_2 are the g values of the two spins, r is the interspin distance, and θ is the angle between the spin−spin vector and the external magnetic field.

There are few reports of distance determinations between two metal centers in native proteins by pulsed ELDOR.[16−18] In a covalently linked azurin dimer model system, X-ray crystallography gave a distance of 2.6 nm between the two Cu^{2+} ions, whereas analysis of pulsed ELDOR spectra led the authors to conclude that the dimer could assume more conformations in frozen solution than in the crystal.[18] Here we extend the methodology to longer distances, using Cu_2Tf and Cu_2Lf as paradigm systems.

Figure 1 depicts the 9 GHz field-swept electron-spin echo spectra of (a) Cu_2Tf and (b) Cu_2Lf. The spectra are characteristic for copper-containing members of the transferrin family.[6,19−24] Each protein contains two Cu^{2+} ions—one bound in each lobe—which have slightly different principal g factors and hyperfine couplings.[21] Analysis of continuous-wave (cw)-EPR spectra under different conditions has shown that they have an approximately axially symmetric g-tensor with $g_{\|} = 2.31$, $g_{\perp} = 2.06$, and $A_{\|} = 16 \pm 2$ mT. The hyperfine coupling to the two copper atoms with their two isotopes ^{63}Cu and ^{65}Cu (natural abundance ratio 69/31, each with a nuclear spin of 3/2) and the hyperfine coupling to an imidazole nitrogen atom from a coordinating histidine has the effect that close to $g_{\perp}$ the contributions from molecules with a wide range of orientations are superimposed, which minimizes the effects of orientation selection at these magnetic fields and microwave frequencies. This is important since, although the ELDOR method involves exciting the spin system at one frequency and detecting an echo at another, spins from all orientations relative to the magnetic field should be sampled to obtain a powder pattern. Hence, we have performed pulsed ELDOR at this position, which also corresponds to the position of maximum intensity.

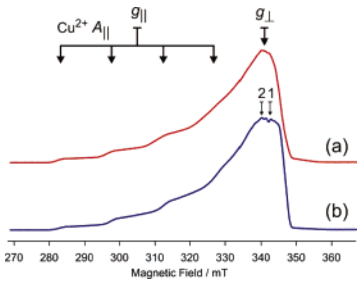

Figure 1. Field-swept electron spin−echo spectra of (a) Cu_2Tf and (b) Cu_2Lf recorded at 10 K with an X-band Bruker ELEXSYS E580 spectrometer operating at 9.845 GHz. A two-pulse echo sequence ($\pi/2-\tau-\pi$) with a 32 ns π-pulse and $\tau = 200$ ns was used. The arrows indicate the positions of pumping (1) and detection (2) in the pulsed ELDOR experiments shown in Figure 2.

In Figure 2a, the four-pulse ELDOR time traces for Cu_2Tf (red) and Cu_2Lf (blue) are shown. Each shows the typical features of pulsed ELDOR spectra: an oscillation superimposed on a decay function. The latter can be fitted using a homogeneous three-dimensional background correction (black lines) as implemented in DeerAnalysis2006 by Jeschke.[25] Following subtraction of the decay function, the curves depicted in Figure 2b are obtained. These curves were fitted using a distance-domain Tikhonov regularization. We note that the fit for Cu_2Tf is poorer than for Cu_2Lf. This is possibly due to a poor baseline correction or a slight orientation selectivity that increases the contribution of the parallel features in the dipolar spectrum. The frequency domain spectra and simulations are shown in Figures 2c,d. The experimental curves are good Pake patterns, implying that our assumption of minimal orientation selection is well-founded. The distance for Cu_2Tf is close to the value of 4.16 ± 0.28 nm obtained by Zweier for CuFeTf.[26] In that study, Fe^{3+} bound at the C-terminal site caused a 0.06 ± 0.01 mT paramagnetic broadening of the cw-EPR spectrum of Cu^{2+} bound at the N-terminal site. The experiment relied on selective insertion of the metal ions into specific binding sites, which is difficult to accomplish as a general method. Both EPR-derived estimates of the distance are 2−3% lower than the values obtained from crystallography, as illustrated by vertical lines in Figure 2e,f. This might reflect a slight conformational difference between the proteins in frozen solution and in a crystal. However, the slow decay of the oscillation is consistent with a narrow distance distribution, 4.16 ± 0.06 nm for Cu_2Tf and 4.24 ± 0.06 nm for Cu_2Lf, indicating that the Tf and Lf structures are more rigid than the azurin dimer.[18]

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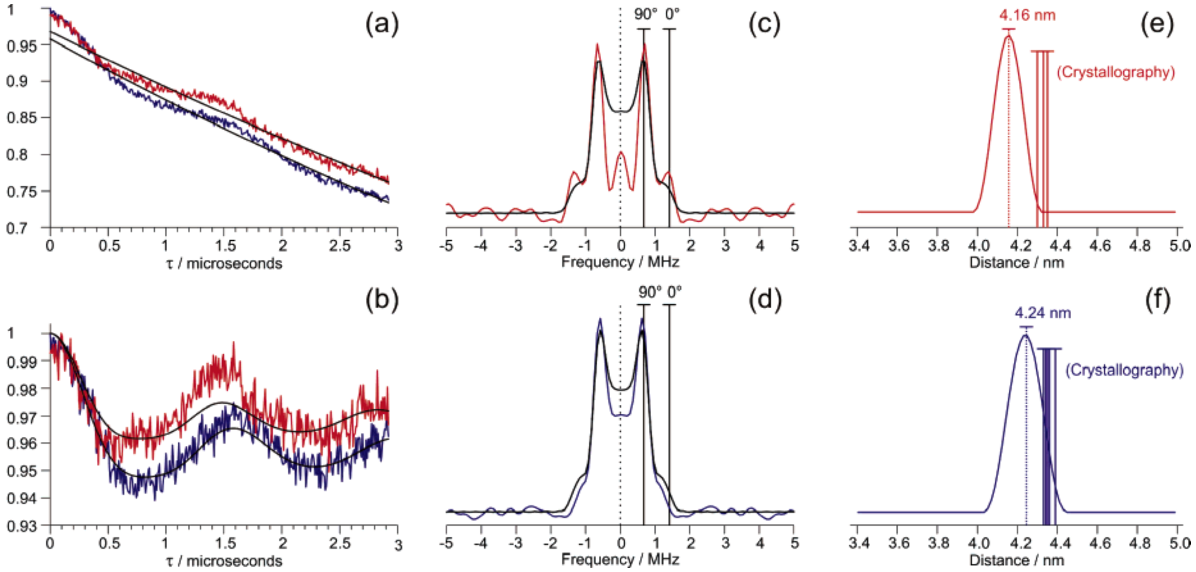

Figure 2. (a) Four-pulse ELDOR spectra and exponential decays of Cu_2Tf (red) and Cu_2Lf (blue) at 10 K. The spectra were recorded close to $g_\perp$ at 341 mT (see Figure 1) with $\nu_{detection} = 9.845$ GHz and $\nu_{pump} = 9.775$ GHz. (b) Time traces and fits of the spectra in (a) after subtraction of the exponential decay. Frequency domain spectra of (c) Cu_2Tf (red) and simulation (black) and (d) Cu_2Lf (blue) and simulation (black). Distance distributions of (e) Cu_2Tf and (f) Cu_2Lf also showing inter-metal distances derived from X-ray structures; see Supporting Information. Analysis was performed using DeerAnalysis2006 by Jeschke. Distances were scaled as the spectra were recorded at $g = 2.06$ instead of $g = 2$ as fixed in DeerAnalysis2006.

To conclude, we have shown that pulsed ELDOR can be used to determine distances greater than 4 nm between copper sites in proteins. This is potentially valuable for cases where proteins can be frozen in states that are not amenable to crystallography, such as membrane-bound complexes or transient states. It will be particularly useful for investigating changes in distances and in distance distributions when, for example, transferrins bind to their bacterial[10] and mammalian[11] receptors.

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Supporting Information Available: Details of sample preparation and X-ray data on Tf and Lf. This material is available free of charge via the Internet at http://pubs.acs.org.

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