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Distances Given by Pulsed EPR

Measurement of a Cu–Cu Distance of 26 Å by a Pulsed EPR Method**

Irene M. C. van Amsterdam, Marcellus Ubbink, Gerard W. Canters, and Martina Huber*

Methods for determining accurate distances in biological and chemical systems are becoming increasingly important. They provide structural information for systems that cannot be crystallized or are otherwise not accessible to the traditional tools of structure determination. In particular, methods are needed to measure long distances to complement existing techniques. In this context, the advantage of electron paramagnetic resonance (EPR) is becoming increasingly apparent since it combines specificity and the potential to measure long distances.^[1,2] Herein we demonstrate that long distances between copper ions in biological systems can be measured by using a pulsed electron electron double resonance (DEER) method.^[3] We have investigated covalently linked dimers of an electron transfer protein, the copper protein azurin—these dimers are model compounds for electron transfer (ET).^[4,5] The models consist of two azurin protein molecules linked through a surface cysteine group introduced by site-directed mutagenesis (N42C mutant); in az-1 directly by a cystine bond (Figure 1) and in az-2 by a flexible linker (1,1'-[oxydi(methylene)]bis(maleimide); BMME).^[4,5]

In the DEER technique, three pulses at the observing frequency ν_{obs} and one pulse at the pump frequency ν_{pump} are combined in the four-pulse sequence shown in Figure 2. With the exception of one unsuccessful attempt to determine the distance between two metal centers in a protein,^[6] previous applications of DEER have been limited to nitroxide spin labels, for which distances of about 30 Å have been determined.^[3]

The importance of reactive metal centers in biological reactions underlines the need to extend the use of the DEER method to paramagnetic transition-metal ions. For the dimers investigated here, ET occurs between the two copper ions, which makes the Cu–Cu distance an important parameter in interpreting the ET rates determined by NMR spectroscopy.^[4,5]

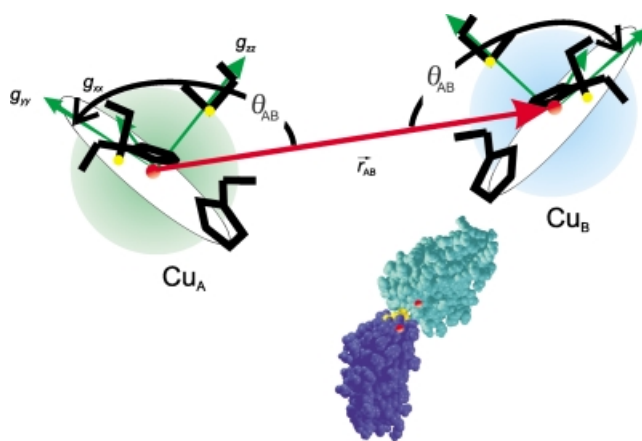


Figure 1. Structure of az-1 dimer. Schematic representation: Relative orientation of the two copper sites Cu_A and Cu_B in the dimer as well as the principal axes of the g tensor with respect to the ligands. $\vec{r}_{AB}$: vector between Cu_A and Cu_B ; θ_{AB} : dipolar angle for B_0 in the g_{xx} – g_{yy} plane. Insert: space-filling model of the dimer structure from X-ray crystallography.^[4]

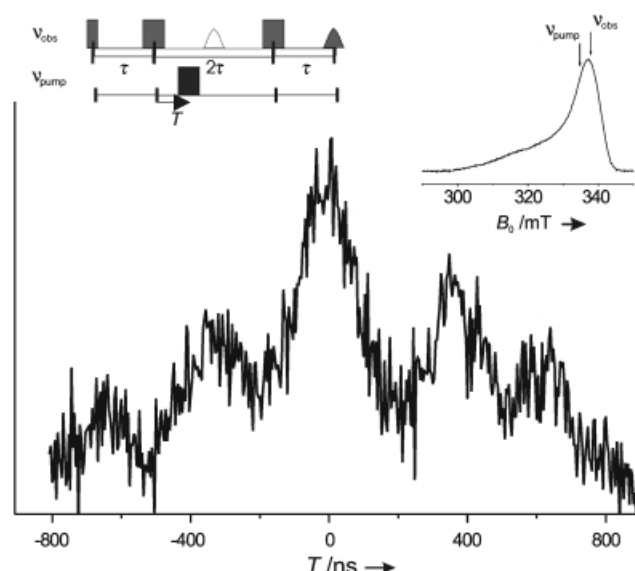


Figure 2. Result of the DEER experiment on az-1, showing the electron spin echo intensity as a function of the time T . The modulation amplitude accounts for about 3% of the total echo intensity. Temperature: 10 K, total measurement time: 75 min. Time increments in T : 4 ns. Insert: DEER pulse sequence (left); echo-detected (absorption type) EPR spectrum (right), with turning points at the principal values of the g tensor $g_{xx}=2.04$, $g_{yy}=2.06$, and $g_{zz}=2.27$.^[8] ν_{obs} : B_0 field at which experiment is performed, ν_{pump} : field corresponding to the frequency offset $\nu_{\text{obs}}-\nu_{\text{pump}}$ of 75 MHz.

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copy.^[4,5] It is not self-evident that Cu^{II} ions can be investigated by the DEER method, since the g - and hyperfine anisotropies of Cu^{II} ions are significantly larger than for nitroxide spin labels. This results in a pronounced dependence of the EPR spectra on the orientation of the magnetic field: only molecules with a particular orientation to the external magnetic field are excited (orientation selection).

Here we show that copper–copper interactions can be measured by the DEER method, and we present a model that

takes into account orientation selection. X-ray crystal structure analysis data for the dimers investigated were available; however, since potential differences between the conformation of the dimers in the crystal and in the (frozen) solution could have an impact on the interpretation of ET rates measured in solution, additional information was sought regarding distances and conformations in solution.

DEER experiments were performed on both dimers. The electron spin echo intensity plotted as a function of the time T for az-1 (Figure 2) shows distinct modulations, which qualitatively agree with those of the nitroxide spin probes.^[3] The Fourier transformation of the signal provides a frequency spectrum (Figure 3a) in which the maximum intensity is observed at the dipolar frequency $\nu_{\text{dip}} = 2.7$ MHz. In the second dimer, az-2, no modulations were observed under the same experimental conditions. The modulations in Figure 2 show that the DEER method can be applied to Cu^{II} centers. In the following we will discuss the frequency spectrum and the differences between the two dimers.

The frequencies reflect the dipolar interaction, which, in the point dipole approximation, is given by Equation (1).

$$\nu_{\text{dip}} = \frac{\mu_0 g_A g_B \beta_e^2}{4\pi h} \frac{1}{r_{AB}^3} (3 \cos^2 \theta_{AB} - 1) \quad (1)$$

It contains r_{AB} , the distance between the paramagnetic centers A and B, g_A and g_B the g values of the two radical

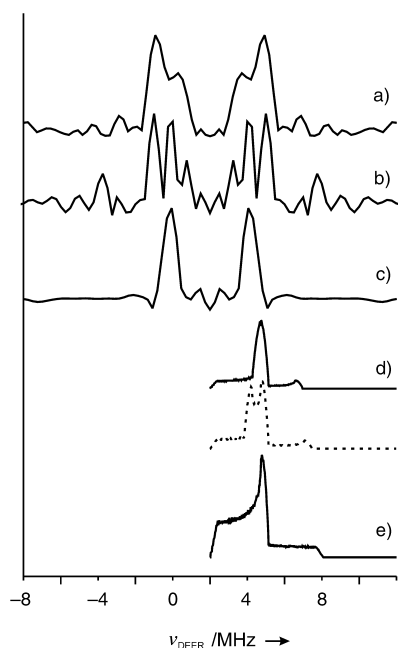


Figure 3. a) Fourier transform of the experimental data (curve from Figure 2). b) Simulation of the powder pattern (e) after inverse Fourier transformation to reconstruct the time trace. These data were reduced to the length of the experimental time trace (1.6 μ s), followed by a Fourier transformation as in a. c) Fourier transform of the sum of simulated dipolar frequencies of Cu_A and Cu_B (d), data manipulation as for b. d) Simulation of dipolar frequencies with orientation selection: Cu_A (solid), Cu_B (dashed); ν_{dip} values calculated for 1° intervals; resonance positions superimposed with Gaussian lines (linewidth 10.0 kHz). e) Simulation of the dipolar spectrum for random orientation.

fragments, and θ_{AB} the dipolar angle of the magnetic field B_0 and the inter-radical vector $\vec{r}_{AB}$ (Figure 1).

The Fourier transform of the DEER response curve for az-1 contains frequencies between 0.7 and 3.6 MHz (Figure 3a), which reflect the distance between the two Cu^{II} centers, r_{AB} , and the dipolar angle θ_{AB} . For random frozen solutions the frequency spectrum of ν_{dip} can be calculated (θ_{AB} between 0° and 90°), which, for example, has been described by Larsen et al.^[7] for nitroxide systems. The simulation of the frequency spectrum for az-1 without orientation selection is shown in Figure 3e. For Cu^{II}, excitation of the EPR spectrum at the positions indicated in Figure 2 selects only those dimers which have a defined orientation relative to the magnetic field. For these orientations B_0 is close to the g_{xx} , g_{yy} plane (Figure 1), effectively reducing the accessible values of θ_{AB} . The range of θ_{AB} is determined by the orientation of the two g tensors relative to $\vec{r}_{AB}$. The orientation of the g tensor with respect to the copper ligand environment is known from high-field EPR spectroscopy on single-crystals of azurin,^[8] and the relative orientation of the two copper centers in the dimer is taken from the X-ray structure analysis of az-1.^[4,5] To calculate the effect of the orientation selection on ν_{dip} we assume that the g tensor is axially symmetric, that is, B_0 is taken to lie in a cone around the unique g_{zz} direction. The orientation of the two copper centers (Cu_A and Cu_B, Figure 1) in the dimer relative to $\vec{r}_{AB}$ is not equivalent, therefore, the frequency distribution of ν_{dip} differs for Cu_A and Cu_B (Figure 3d). Coincidentally, the orientation of the two copper centers in az-1 is such that the most abundant orientations cause frequencies close to the maximum in the powder pattern for the same distance (Figure 3e). The simplified model neglects an additional orientation selection, which is imposed by the fact that only those molecules show modulation in which Cu_A is excited at ν_{obs} and Cu_B at ν_{pump} and vice versa.^[7] Therefore, the actual frequency distribution is expected to be narrower than the one calculated by using the model. However, the observed spectrum (Figure 3a) is broader than the calculated one (Figure 3d). This could be a result of the limited time window T over which the modulation was measured. To test this, the frequency distribution of the simulation was treated in the same way as the experimental data (see caption for Figure 3). This causes distinct changes in the lineshape (Figure 3c). After this treatment the experimental spectrum (Figure 3a) is broader than the simulated spectrum (Figure 3a). It is less broad, however, than the simulated spectrum obtained by the same method from the simulation of a random sample (Figure 3b). Thus we conclude that the dimer can assume more configurations in frozen solution than in the crystal, without, however, resulting in a random orientation.

The absence of a modulation for the dimer az-2 is attributed to the shorter copper-copper distance ($r_{AB} = 25.9$ Å (az-1) and 14.6 Å (az-2))^[4,5] resulting in a larger dipolar interaction between the centers (az-1: $\nu_{\text{dip}} = 3.0$ MHz, az-2: $\nu_{\text{dip}} = 17.2$ MHz for $\theta_{AB} = 90^\circ$, see [Eq. (1)]). The ν_{dip} value for az-2 is larger than the experimental excitation bandwidth (see Experimental Section), which explains the absence of modulations for az-2.

In summary, the results of the measurements and the simulations demonstrate that orientation selection affects the DEER response. For az-1 it suggests that in solution more conformations are accessible than in the crystal, an aspect which is currently undergoing detailed analysis. With respect to the determination of distances in other systems, usually enough information about the relative g tensor orientation is known to allow the distances to be determined with sufficient accuracy. If the relative orientation is not known, the dipolar frequency ν_{dip} will differ by a factor of 2 between the two extreme angles $\theta_{\text{AB}} = 0^\circ$ and 90° . The resulting difference in r_{AB} of 25% is therefore the maximum uncertainty expected from orientation effects. Finally, the study demonstrates that DEER can be applied to the determination of structures of compounds which contain paramagnetic transition-metal ions, and thus provides an additional method for structural investigations on biological systems. This is particularly important given the physiological significance of metal ions.

Experimental Section

Spectra were measured on a Bruker Elexsys E680 spectrometer with modifications to the microwave bridge to introduce the second microwave frequency analogous to those described by Pannier et al.^[3] using a HP synthesizer HP 83752B and a microwave amplifier (Microwave Amplifiers AL 16-9-10-15). The four-pulse DEER sequence^[3] with pulse lengths of 16 ns for $\pi/2$, 32 ns for π pulses, and $\tau = 896$ ns was employed. Pulse powers were adjusted to equal intensity at ν_{obs} and ν_{pump} . The maximum frequency separation of ν_{obs} and ν_{pump} was estimated by detuning the measurement frequency from the resonator frequency. At offsets of ± 37.5 MHz attenuation of the signal was observed, leading to the choice of $\nu_{\text{obs}} - \nu_{\text{pump}}$ of 75 MHz. At that separation, the echo is reduced by less than a factor of 2. The excitation bandwidth of the pulses is smaller than in the experiments described by Pannier et al., where a maximum of 25 MHz is given.^[3] In the Fourier transformation (by using the Origin program (Microcal(TM) Northampton, USA) of the modulation, a Gaussian background was subtracted from the experimental data, the resulting curve was filtered with a Hamming window, and filled with zeros to 1024 points to a total time of 3.2 μ s.

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Propylene Oxide Polymerization

An Efficient Method for Controlled Propylene Oxide Polymerization: The Significance of Bimetallic Activation in Aluminum Lewis Acids**

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Propylene oxide (PO) is with a worldwide production of 4.5 million tons a significant intermediate of the petrochemical industry. Two-thirds of the propylene oxide is converted by ring-opening polymerization into polypropylene glycols and propoxylated products of polyurethane.^[1] The classical initiator for this reaction is KOH, whose high basicity leads to deprotonation of the methyl group, resulting in side reactions.^[2] Rapid and controlled PO polymerization has recently become possible through so-called double-metal cyanide compounds (DMC), whose structure and mechanism is, however, still unclear.^[3] Only a small number of structurally characterized initiators for living coordination PO polymerization has been published so far: aluminum complexes with tri- or tetradentate ligands (porphyrins, phthalocyanine, tetraazaannulene, salen-type ligands, diethylenetriamine) polymerize PO by a mechanism based on chain growth at a single metal center by reaction of the monomer with the coordinated alkoxo ligand (in addition, *cis* migration, rear attack, and participation of two metal centers are also assumed).^[4] The sterically demanding Lewis acidic organo-aluminum complexes accelerate the coordination ring-opening polymerization.^[5] We report here the targeted synthesis of new aluminate complexes and their polymerization properties in combination with their neutral Lewis acid precursors. Our results prove for the first time that PO polymerization does not occur at a single Lewis acidic metal center and confirm the earlier proposal of bimetallic activation by Price and Vandenberg.^[6,7]

$[\{\text{Al}(\text{L})\text{Cl}\}_2]$ (**1**: L = mbmp, **2**: mmcp), easily obtained by reaction of AlEt_2Cl with 2,2'-methylenebis(6-*tert*-butyl-4-methylphenol) (mbmpH₂) or 2,2'-methylenebis(4-methyl-6-(1-methylcyclohexyl)phenol) (mmcpH₂), as well as the isopropanolato complexes $[\{\text{Al}(\text{L})(\mu\text{-O}i\text{Pr})\}_2]$ (**3**: L = mbmp, **4**: mmcp), obtainable from trimethylaluminum in a two-step procedure, did at first appear to be suitable for ring-opening polymerization of PO (Scheme 1).^[8,9] With these initiators we could, however, only observe slow (> 24 h) and regioirregular oligomerization of PO.^[10,11] We suspect that the ring opening

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