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Multifrequency Pulsed Electron Paramagnetic Resonance on Metalloproteins

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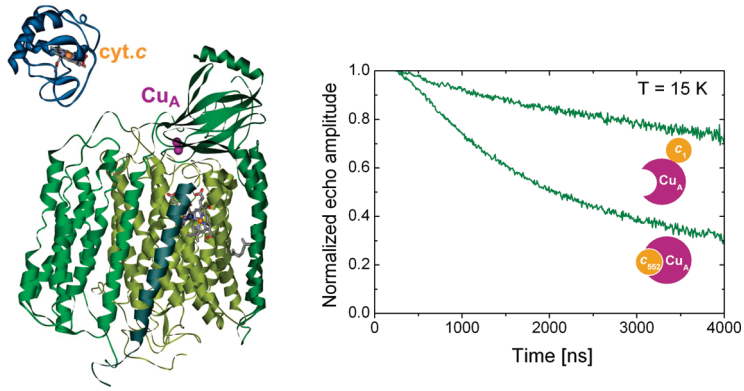

Metalloproteins often contain metal centers that are paramagnetic in some functional state of the protein; hence electron paramagnetic resonance (EPR) spectroscopy can be a powerful tool for studying protein structure and function. Dipolar spectroscopy allows the determination of the dipole−dipole interactions between metal centers in protein complexes, revealing the structural arrangement of different paramagnetic centers at distances of up to 8 nm. Hyperfine spectroscopy can be used to measure the interaction between an unpaired electron spin and nuclear spins within a distance of 0.8 nm; it therefore permits the characterization of the local structure of the paramagnetic center's ligand sphere with very high precision. In this Account, we review our laboratory's recent applications of both dipolar and hyperfine pulsed EPR methods to metalloproteins.

We used pulsed dipolar relaxation methods to investigate the complex of cytochrome *c* and cytochrome *c* oxidase, a noncovalent protein−protein complex involved in mitochondrial electron-transfer reactions. Hyperfine sublevel correlation spectroscopy (HYSCORE) was used to study the ligand sphere of iron−sulfur clusters in complex I of the mitochondrial respiratory chain and substrate binding to the molybdenum enzyme polysulfide reductase. These examples demonstrate the potential of the two techniques; however, they also highlight the difficulties of data interpretation when several paramagnetic species with overlapping spectra are present in the protein. In such cases, further approaches and data are very useful to enhance the information content.

Relaxation filtered hyperfine spectroscopy (REFINE) can be used to separate the individual components of overlapping paramagnetic species on the basis of differences in their longitudinal relaxation rates; it is applicable to any kind of pulsed hyperfine or dipolar spectroscopy. Here, we show that the spectra of the iron−sulfur clusters in complex I can be separated by this method, allowing us to obtain hyperfine (and dipolar) information from the individual species. Furthermore, performing pulsed EPR experiments at different magnetic fields is another important tool to disentangle the spectral components in such complex systems. Despite the fact that high magnetic fields do not usually lead to better spectral separation for metal centers, they provide additional information about the relative orientation of different paramagnetic centers. Our high-field EPR studies on cytochrome *c* oxidase reveal essential information regarding the structural arrangement of the binuclear Cu_A center with respect to both the manganese ion within the enzyme and the cytochrome in the protein−protein complex with cytochrome *c*.

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Introduction

Metalloproteins are heaven for electron paramagnetic resonance (EPR) spectroscopy: many of them contain metal centers that are paramagnetic in some functional state of the protein. Therefore, EPR spectroscopy is the method of choice to identify such states and obtain functional information. Furthermore, deeper understanding of the metalloprotein function and structure can be achieved via characterization of properties such as redox state and ligand geometry for different functional states of the protein. This information was the basis of the understanding of numerous reactions catalyzed by such proteins.

Metalloproteins also may be hell for EPR spectroscopy: often many paramagnetic centers are involved, leading to strong spectral overlap and thus complicating the detailed analysis and description of the single centers. In addition, most paramagnetic metal centers exhibit very broad spectra and extremely fast relaxation times, posing serious restrictions to the application of pulsed methods.

Nevertheless, EPR spectroscopists have learned to handle these restrictions and adapted or extended a variety of methods in order to apply them to metal centers in proteins. Continuous wave (cw) EPR spectroscopy has allowed identification of functional states of metal centers in many metalloproteins.[1] cw-ENDOR (electron nuclear double resonance) spectroscopy has been successfully applied to characterize the ligand sphere of such centers.[2,3] The proper choice of the magnetic field strength and temperature (typically 2−100 K) was used to partially disentangle spectral components from different centers.

More recently, pulsed methods have been employed for paramagnetic centers in metalloproteins[4] to determine dipolar[5−9] and hyperfine[10−16] couplings to nearby electron or nuclear spins. In this Account, we restrict ourselves exclusively to these methods and focus on the possibility of unraveling spectra from different paramagnetic species, either based on their relaxation times or by application of multifrequency EPR to obtain unique structural information from EPR spectroscopic data.

EPR Methods

Due to the very short electronic spin relaxation times of metal ions, EPR spectroscopical methods on metal centers in proteins are commonly applied at low temperatures (liquid helium or liquid nitrogen). Hence, it is not possible to follow catalytic reactions in real time. However, intermediate functional states can be trapped and preserved in frozen solution.[17−19] The orientation-dependent anisotropies of the spin interactions (hyperfine and **g**-tensor) for such centers are usually much larger than the inverse rotational correlation time of the protein. Even at room temperature, the anisotropies are not averaged out, resulting in broad powder spectra similar to solid-state NMR spectroscopy. Thus, EPR spectroscopy is not limited by the size of the protein and can even be applied to proteins embedded in membranes, if spin concentrations of about 10−100 μM can be achieved.[20]

Analyzing the specific line shape of these powder shapes enables identification of paramagnetic species; however, in most cases the interactions with the surroundings are not resolved. There are two important interactions that allow structural information about the paramagnetic center within the protein to be obtained. Both are magnetic dipole−dipole interactions, either with a nonzero nuclear spin or with other paramagnetic moieties in the protein. Since the magnetic moment of the electron spin is several orders of magnitude larger compared to the magnetic moment of the nucleus (e.g., 660 for ^{1}H), respective methods are rather different. *Hyperfine spectroscopy*[10] accounts for interaction with nuclear spins, and *dipolar spectroscopy*[5] reflects interactions with other paramagnetic centers. Also, the detectable distance range of these interactions is different for the same reason: usually electron−nuclear interactions can be observed within a radius of up to 0.8 nm from the paramagnetic center, whereas electron−electron interactions range up to 8 nm.

Hyperfine Spectroscopy. In order to observe an interaction with a paramagnetic center nuclei have to possess a nuclear magnetic moment. These nuclei can be of naturally abundant isotopes (like ^{1}H, ^{14}N, or ^{31}P) or of enriched isotopes (e.g., ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{33}S).[12,13,21,22] The large magnetic moment of the nearby paramagnetic center causes a strong additional local field at these nuclei, which is not small compared to the external magnetic field (0.3 T/9 GHz (X-band)). This leads to mixed spin states and complicates the analysis of the experimental data. In such cases, these interactions can be observed in coherent 1D and 2D pulsed hyperfine experiments, such as ESEEM (electron spin echo envelope modulation)[10] and HYSCORE (hyperfine sublevel correlation spectroscopy).[23] In ESEEM and HYSCORE measurements, the external magnetic field can be optimized to obtain the strongest mixing of nuclear spin states and therefore the largest modulation depth.[24] Performing experiments at higher magnetic fields, such that the nuclear Zeeman splitting is much larger than the hyperfine coupling, strongly reduces the mixing of nuclear eigenstates. Thus, high-fields are optimal for performing ENDOR experiments, which rely on pure eigenstates. Furthermore, at high magnetic fields, the nuclear Larmor

frequencies will be better separated, further simplifying the analysis of ENDOR data.[25,26]

Dipolar Spectroscopy. Measurement of the dipolar coupling strength between spatially separated paramagnetic species is called dipolar spectroscopy.[5] For distances exceeding 1−2 nm, pulsed methods such as pulsed electron−electron double resonance (PELDOR)[27−29] or double quantum coherence (DQC) EPR[30] are superior to cw-EPR methods: inhomogeneous linewidth contributions are suppressed, and coherent time domain detection of dipolar couplings is possible. Both methods work best for paramagnetic centers with rather narrow EPR spectra and long transversal relaxation times. For most metal ions in enzymes, only noncoherent dipolar relaxation methods can be utilized to determine the distances between them.[5] Owing to the statistical nature of the relaxation process, the dipolar coupling will only lead to an enhanced decay of the signal.[31] Thus, relaxation measurements are mostly applied in a more qualitative manner compared with the above-mentioned coherent experiments.

High-Field EPR and Relaxation-Filtered Hyperfine (REFINE) Spectroscopy. In complex systems containing several paramagnetic species, information gathered by EPR spectroscopy at X-band is usually not sufficient to unambiguously deduce structural and chemical properties of the paramagnetic states under study. In these cases, the separation and assignment of EPR signals, hyperfine and dipolar contribution to the different paramagnetic centers, can be strongly improved by performing EPR experiments at different magnetic field strengths or at different microwave frequencies, respectively.[11,14,32,33] While multifrequency pulsed EPR experiments allow unambiguous disentanglement of hyperfine and dipolar contributions due to the field dependence of the nuclear Zeeman interaction,[34] high-field EPR increases the spectral resolution, thus leading to unique spectroscopic assignments.[35−38] In addition, the orientation selectivity achieved at high magnetic fields can be used to obtain angular information.[39−41]

Spectral overlap of different paramagnetic species poses severe problems to the assignment of hyperfine lines to the individual species. For metal centers, high-field EPR will not improve the situation because the spectral shape is caused by the anisotropy of the **g**-tensor. A classical way to discriminate between the contributions of different metal ions is by temperature variation, where the strong temperature dependence of the relaxation behavior is used.[1] This method has two drawbacks: only the slowest relaxing species can be individually observed, and because of the elevated temperature, they can only be observed at a strongly reduced sensitivity. However, it is possible to separate species via their different longitudinal relaxation time using pulsed EPR methods such as REFINE (relaxation filtered hyperfine spectroscopy).[42−44] In this method, an additional preparation pulse sequence encodes the hyperfine spectrum of each paramagnetic center with its individual relaxation behavior. This allows unraveling of paramagnetic species with distinguishable relaxation times by a systematic variation of the time delay T between the preparation and detection sequence. This method can also be applied to dipolar spectroscopy, if more than two paramagnetic species with different longitudinal relaxation times are involved, such as FeS centers in electron transfer proteins.

Applications

Here, examples of the application of hyperfine and dipolar spectroscopy to metalloenzymes from our own laboratory are sketched to demonstrate the potential and limitations of pulsed EPR methods on such systems. Special emphasis will be placed on the possibility of separating overlapping spectra from different paramagnetic centers by high-field and relaxation-based methods.

Substrate Binding to a Molybdoenzyme Studied by HYSCORE Spectroscopy. Polysulfide reductase (Psr) is an essential enzyme for sulfur respiration of the anaerobic bacterium *Wolinella succinogenes*.[45] Biochemical and spectroscopic studies have shown that Psr resembles enzymes of the DMSO reductase family,[46] carrying a mononuclear molybdenum center, coordinated by two molybdopterin guanine dinucleotides (MGD) and a cysteine amino acid residue, five iron−sulfur (FeS) clusters, and a methyl-menaquinone as redox-active cofactors.[45] It has been suggested that the molybdenum center is the active site of this enzyme, catalyzing the reduction of the substrate polysulfide to sulfide. Multifrequency cw-EPR studies have revealed three spectroscopically distinct Mo(V) functional states of Psr based on the Mo(V) hyperfine- and **g**-tensor values.[47] The state generated by addition of polysulfide (very-high-G) has been proposed to be the catalytically active form, in which Mo is coordinated by a sulfur of the polysulfide chain as the sixth ligand.

Here, ^{33}S ($I = {}^3/_2$) isotope-labeled polysulfide was prepared in order to probe the proximity of the polysulfide to the Mo(V) center via its hyperfine interaction and thus to unambiguously determine the identity of the active site and the substrate. Pulsed hyperfine spectroscopy (HYSCORE) has been used to detect these hyperfine and quadrupole interactions, which were unresolved in cw-EPR spectra.[12,19,21] The X-band HYSCORE spectra of Psr samples prepared with ^{33}S-labeled polysulfide or polysulfide containing the naturally abundant

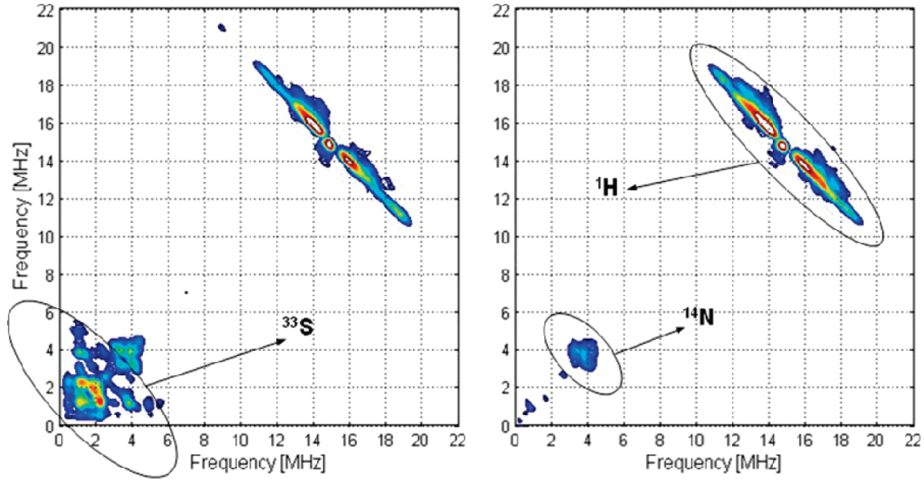

FIGURE 1. HYSCORE spectra of the Mo(V) very-high-G state of Psr generated with ^{33}S-labeled (left) and unlabeled polysulfide (right). Spectra are taken at a field position corresponding to g_{yy} of the Mo(V) signal.

^{32}S isotope performed at the center of the Mo(V) signal are depicted in Figure 1. Strong additional cross-correlation peaks at low frequencies were resolved for Psr treated with ^{33}S polysulfide, verifying that a sulfur nucleus from the polysulfide substrate is indeed in close vicinity to the molybdenum active site. The shown HYSCORE spectrum exemplifies the power of this 2D method to disentangle complex hyperfine information arising from different paramagnetic states and nuclei. The additional spectral features in the frequency range 3−5 MHz observed in both samples originate from backbone nitrogens coupling to the FeS centers in Psr (cross-peaks not well resolved on the chosen contour level). The off-diagonal ridges, situated symmetrically at the ^{1}H Larmor frequency, result from hyperfine couplings of the Mo(V) unpaired electron spin with nearby protons. The experimental observations strongly indicate that the ^{33}S is indeed the sixth ligand of the Mo(V) center and that polysulfide is the actual substrate for this enzyme.

In this example, 2D-HYSCORE hyperfine spectroscopy and the optimal choice of experimental temperature was sufficient to separate the contributions from the different paramagnetic species. However, the next example demonstrates that this is not always possible.

Hyperfine Characterization of FeS Centers in Complex I by REFINE. NADH:ubiquinone oxidoreductase (complex I, 1 MDa), the first complex of the mitochondrial respiratory chain is among the largest and most complicated membrane-bound multiprotein complexes known.[48,49] It links the electron transfer from NADH to ubiquinone to the concomitant translocation of four protons across the inner membrane.[50] Because of its central role in respiration, mutations in complex I can lead to numerous human disorders[51] and have been suggested to be a major source of reactive oxygen species (ROS) in mitochondria.[52]

Complex I has an L-shaped structure, with the hydrophobic arm embedded in the membrane and the hydrophilic peripheral part protruding into the mitochondrial matrix or the bacterial cytoplasm.[53] It contains several cofactors, a noncovalently bound flavine mononucleotide (FMN), and, depending on the organism, up to nine FeS clusters.[54−56]

The obligate aerobic yeast *Yarrowia lipolytica* is a powerful model system for the structural and functional analysis of complex I.[57] EPR spectra of complex I of *Y. lipolytica* in its NADH-reduced state are similar to those from bovine heart[55] and show five FeS clusters, designated N1−N5.[57]

The temperature dependence (5−30 K) of complex I EPR spectra is shown in Figure 2. At 30 K, only cluster N1 is visible, while at lower temperature, additional FeS cluster signals appear, and at 5 K, four FeS clusters are visible.[58] Besides the number of paramagnetic centers, the informational content of the field-sweep spectrum is limited. However, ESEEM and HYSCORE can be employed to reveal the hyperfine interactions hidden within the inhomogeneous linewidth and yield information about the local environment of the paramagnetic center.

At 30 K, cluster N1 can be investigated individually, and three-pulse ESEEM and HYSCORE experiments performed at the $g_{\perp}$ and $g_{\parallel}$ components of the **g**-tensor reveal a hyperfine interaction of cluster N1 with a single ^{14}N nucleus (Figure 3), typical for a ferrodoxin-type coordination (Figure 3, right bottom). This finding was later confirmed by the crystal structure.[55]

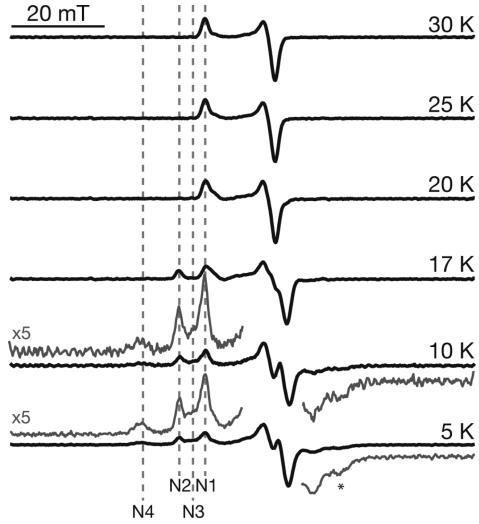

FIGURE 2. EPR spectra (9 GHz) of complex I from *Y. lipolytica* at different temperatures. The $g_{||}$ components of the FeS clusters N1−N4 are indicated by dashed lines (cluster N5 is not detectable under the conditions used here). Figure adapted from ref 58.

In complex I, cluster N2 plays an important role in the redox-linked proton translocation,[59] and the nature of the fourth ligand was unknown for a long period of time. Two nitrogen-containing amino acid residues (arginine or histidine) were proposed as possible candidates to ligate cluster N2.[60] However, while cluster N1 can be studied separately at 30 K, this is impossible for cluster N2 due to the severe overlap of the EPR spectra of both clusters (Figure 4, top left). In this case, inversion−recovery filtered (REFINE) spectroscopy can be utilized.[42,43]

The filtered spectra of complex I taken at 17 K are shown in Figure 4 (top panel). Using a filter time of $T_F = 68$ or 420 ns, one can unravel the contribution of cluster N1 and N2 with respect to the overall EPR spectrum. These filter times have been used in a REFINE−ESEEM experiment to study the hyperfine interactions of each FeS cluster discretely (Figure 4, middle and bottom panel). It has been concluded that only cluster N1 shows an interaction with an ^{14}N nucleus since the ESEEM spectrum at $T_F = 68$ ns is similar to the spectrum taken with a filter time $T_F = 50$ μs, at which the system is back at the thermal equilibrium.

Here, REFINE spectroscopy was used to assign the nitrogen hyperfine couplings individually to N1 and N2. Furthermore, REFINE experiments can also be employed to separate more than two overlapping species, by introducing a further dimension that encodes the relaxation behavior of the individual paramagnetic components.[44] An application of REFINE to complex I to spectrally separate all five FeS centers and to determine their dipolar coupling network is in progress in our laboratory.

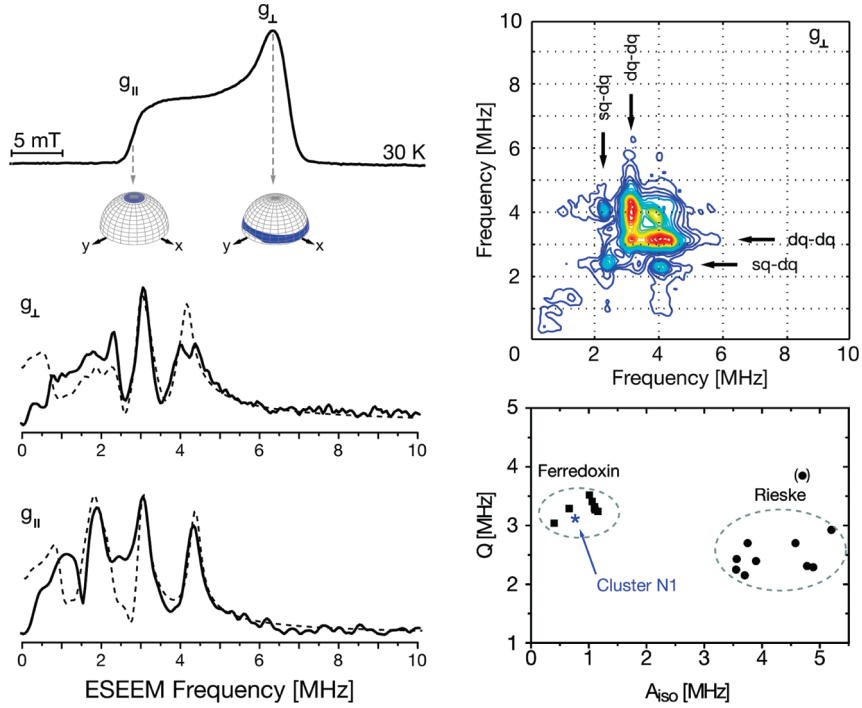

FIGURE 3. Field-sweep spectrum (left, top) of cluster N1 at 30 K, with principal tensor components $g_\perp$ and $g_{||}$ indicated (excited orientations are shown below), three-pulse ESEEM spectra (left, bottom) taken at magnetic field positions corresponding to $g_\perp$ and $g_{||}$ (solid line, simulations shown as dashed line), HYSCORE spectrum (right, top) taken at a magnetic field position corresponding to $g_\perp$, and hyperfine and quadrupole parameters (right, bottom) of ^{14}N ligands of [2Fe−2S] clusters of different metalloproteins and cluster N1. Figure adapted from ref 58.

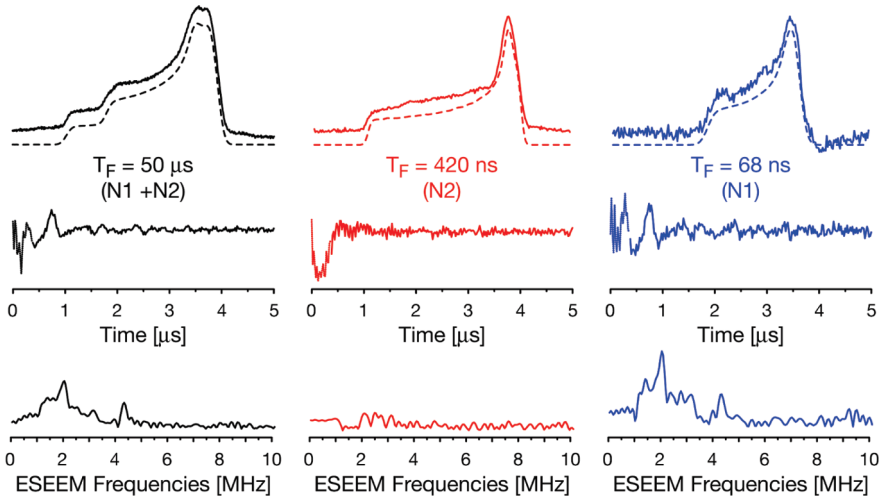

FIGURE 4. Inversion–recovery filtered field-swept EPR spectra (top panel) of *Y. lipolytica* complex I at 17 K and filter times indicated (simulations shown as dashed lines), REFINE-ESEEM time traces (middle panel), and Fourier transform of the time traces (bottom panel). Figure adapted from ref 42.

High-Field EPR Characterization of the Cu_A–Mn Dipolar Interaction in Cytochrome *c* Oxidase. Cytochrome *c* oxidase (CcO) performs the final step in the electron transport chain of mitochondria and many aerobic bacteria. It catalyzes the transfer of four electrons from reduced cytochrome *c* to molecular oxygen, forming two water molecules.[61,62] CcO carries four redox-active metal centers: a binuclear copper center (Cu_A) in subunit II and a low-spin heme *a*, a high-spin heme a_3, and a mononuclear copper center (Cu_B), all in subunit I.[63] In addition, a redox-inactive metal binding site located between subunits I and II is found. This site could bind Mn as well as Mg; however its function remains unknown. The arrangement of the redox-active cofactors in the crystal structure of CcO from *Paracoccus denitrificans*[63] is shown in Figure 5.

The distance and exchange interaction between the Mn^{2+} ion and the mixed-valence Cu_A center in CcO from *P. denitrificans* has been determined using multifrequency cw-EPR.[34] In particular high-field EPR allowed separation of the Mn^{2+} hyperfine lines from the EPR signals of the other paramagnetic centers and thus observation of the dipolar splitting due to the interaction with the paramagnetic Cu_A center in the oxidized form of the protein. Such a splitting is not present in the reduced enzyme, where Cu_A is diamagnetic (Figure 6).

The splitting on the Mn^{2+} lines into doublets could be quantitatively described by purely dipolar spin–spin interaction between the paramagnetic Mn^{2+} and the binuclear Cu_A center. The calculated Mn^{2+}–Cu_A distance of 9.4 Å in the fully oxidized protein is in good agreement with the X-ray structure. The high-field EPR data do not support structural modifications in the Mn/Mg binding site within the redox cycle, as has been postulated from X-band EPR experiments.[64] This confirmed that the Mg/Mn site does not participate in the electron transfer process.

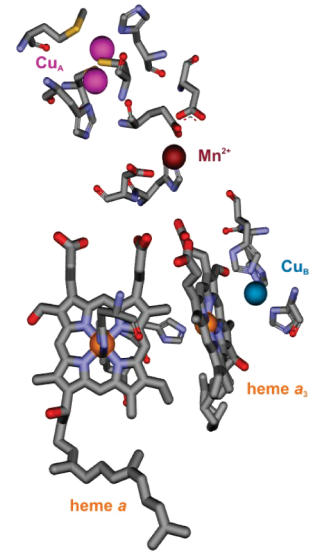

FIGURE 5. Metal centers in subunits I and II of CcO from *P. denitrificans* with their corresponding amino acid ligands.

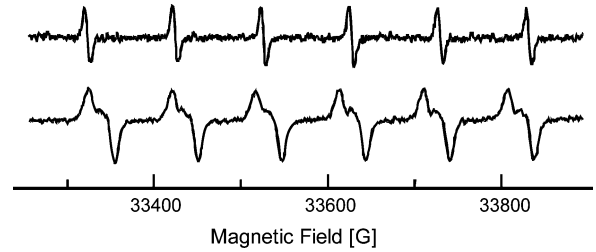

FIGURE 6. W-band (95 GHz) spectra of oxidized (bottom) and reduced (top) CcO. Figure adapted from ref 34.

Multifrequency Relaxation Study of the Complex between Cytochrome *c* and Cytochrome *c* Oxidase. Specific protein–protein recognition is an important step in bioen-

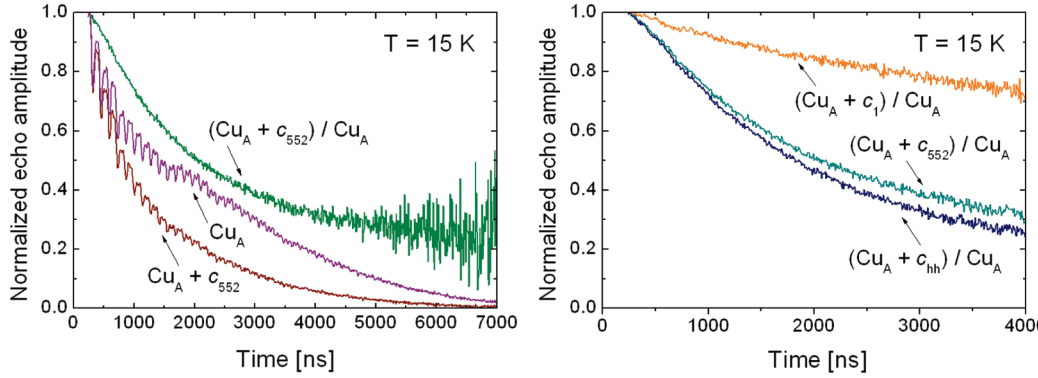

FIGURE 7. Electron-spin echo (9 GHz) decay traces (left) of the soluble Cu_A domain of CcO_{II} alone (Cu_A) and in a mixture with cytochrome c_{552} ($Cu_A + c_{552}$). Division of these two time traces yields a pure dipolar relaxation trace ($Cu_A + c_{552}$)/Cu_A. Dipolar relaxation traces (right) of mixtures of CcO_{II} with horse heart cytochrome c_{hh} (($Cu_A + c_{hh}$)/Cu_A), c_{552} (($Cu_A + c_{552}$)/Cu_A), and cytochrome c_1 (($Cu_A + c_1$)/Cu_A). Figure adapted from ref 67.

ergetic processes, such as respiration and photosynthesis, where protein interactions are known to be of transient nature.[65] Within bacterial and mitochondrial respiratory chains, several interactions are required such as that between CcO and cytochrome *c*.[66] Since both of these electron transfer proteins carry paramagnetic centers (the binuclear Cu_A center as the first electron acceptor in CcO and the heme of cytochrome *c*), protein−protein interactions can be studied by dipolar EPR spectroscopy. Paramagnetic metal ions are characterized by extremely broad spectral line widths and very short relaxation times; hence, the method of choice for metalloproteins is dipolar relaxation enhancement.[5,8]

Two-pulse electron spin echo experiments have been carried out on mixtures of the Cu_A-containing soluble fragment of subunit II of CcO (CcO_{II}) with cytochrome *c*, where Cu_A is the slowly relaxing observer spin and Fe(III) of cytochrome *c* is the fast relaxing spin.[67] Relaxation enhancement upon protein−protein binding has been observed (Figure 7, left panel). Division of the time traces in the presence and absence of cytochrome *c* allowed us to extract the dipolar relaxation. Such dipolar relaxation curves for interactions with different *c*-type cytochromes are depicted in Figure 7.

Significantly enhanced relaxation of Cu_A due to transient protein−protein recognition has been observed for two specifically interacting cytochromes: the physiological partner cytochrome c_{552} and horse heart cytochrome *c* (c_{hh}). In contrast the nonbinding cytochrome c_1 showed only a very weak relaxation enhancement due to nonspecific protein−protein interactions.

Experiments at temperatures in the range of 12−23 K proved the dipolar nature of the relaxation enhancement (Figure 8) and revealed a broad distribution in distances (2−4 nm) and orientations between the Cu_A and Fe(III), supported by the

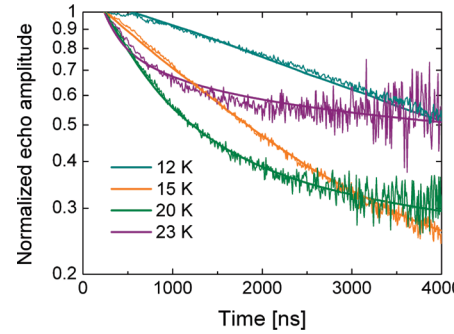

FIGURE 8. Dipolar relaxation traces of mixture of CcO_{II} with c_{hh} at different temperatures (simulation shown as solid line). Figure adapted from ref 67.

lack of relaxation anisotropy in the orientation-selective experiments performed at 180 GHz (G-band).[67]

This finding suggests that the protein−protein complex between CcO and cytochrome *c* can not be described by a single well-defined structure, in agreement with a computational docking study.[68]

Summary and Outlook

Pulsed EPR methods can be used to address specific questions on metal centers in proteins. Hyperfine spectroscopy is the method of choice to study the local structure of metal centers, since it reveals information on the type of nuclei in the vicinity of the paramagnetic center, the distance, and the geometry. Dipolar spectroscopy opens new possibilities to determine distances between paramagnetic metal centers involved in electron transfer or catalytic reactions. Importantly, pulse and high-field EPR methods may be employed to separate spectrally overlapping paramagnetic species, a situation rather typical in metalloproteins, as illustrated by some of our recent applications in this Account.

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FOOTNOTES

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