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NMR Study of the Exchange Coupling in the Trinuclear Cluster of the Multicopper Oxidase Fet3p

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Abstract: Fet3p from *Saccharomyces cerevisiae* is a multicopper oxidase (MCO) which oxidizes Fe^{2+} to Fe^{3+}. The electronic structure of the different copper centers in this family of enzymes has been extensively studied and discussed for years with a particular focus on the exchange coupling regime in the trinuclear cluster (TNC). Using NMR spectroscopy we have quantified the exchange coupling constant in the type 3 center in a fully metalated oxidase; this value in Fet3p is significantly higher than that reported for proteins containing isolated type 3 centers as tyrosinase. We also provide evidence of exchange coupling between the type 2 and the type 3 Cu^{2+} ions, which supports the crystallographic evidence of dioxygen binding to the TNC. This work provides the foundation for the application of NMR to these complex systems.

Introduction

Copper centers are employed by proteins to perform electron transfer and redox chemistry, primarily oxygen activation.[1−5] Multicopper oxidases (MCOs) are a ubiquitous family of enzymes that couple the reduction of O_2 to H_2O with the oxidation of specific substrates.[4,6−8] These enzymes are present in eukaryotes, bacteria, and archea, playing crucial roles in lignin degradation and synthesis, copper homeostasis, and iron metabolism.[4,6,7] MCOs can be divided into two groups based on their substrate specificities; most of them catalyze the oxidation of organic substrates (laccases and ascorbate oxidase)[9,10] while others oxidize transition metal ions (Fet3p, ceruloplasmin, CueO, MnxG).[11−13] Substrate oxidation and oxygen reduction are performed by copper ions in different sites. MCOs contain at least four copper ions, which are arranged in a type 1 (T1, blue) mononuclear copper center and a trinuclear copper cluster (TNC) consisting of a type 2 (T2) copper ion and a binuclear type 3 (T3) center (Figure 1).[7] The substrate is oxidized by the T1 site, which then shuttles electrons to the trinuclear cluster, where the dioxygen reduction takes place. Thus, the T2 and the T3 centers represent a functional unit in which the T3 site exhibits unique electron transfer activity.[9,14]

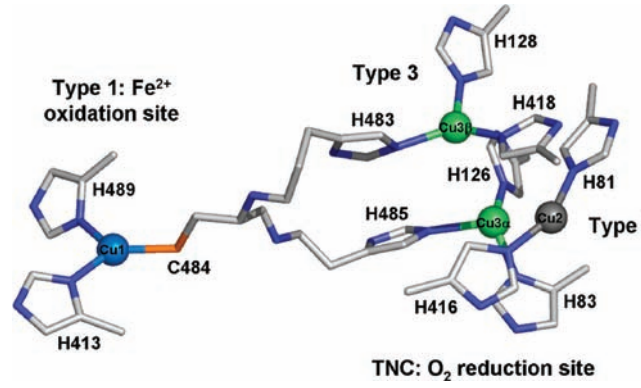

Figure 1. Multinuclear metal site of reduced Fet3p (PDB accession code 1zpu). Type 1, type 2, and type 3 copper ions are shown as blue, gray, and green spheres, respectively. The figure was rendered with PyMol version 0.97 (DeLano Scientific).

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Copper centers have been historically defined according to their spectroscopic features.[15] These features have been interpreted to reflect the required electronic structure of these centers to fulfill their biological roles. T1 and T2 centers are both mononuclear but can be differentiated by their distinct functional roles; T1 sites are involved in intra- and intermolecular electron transfer processes, while T2 centers normally perform redox chemistry. Binuclear T3 copper centers are present in many different proteins, and their roles involve dioxygen binding, activation, and reduction.[5] They can be found in proteins which possess only binuclear T3 Cu sites, such as tyrosinases and catechol oxidases (both involved in the oxidation of organic substrates)[16] or hemocyanins (molecular oxygen carriers which reversibly bind O_2).[5] In MCOs, the T3 center is part of the trinuclear cluster which constitutes a single reactive unit.[9,14] Despite their very different chemistries, type 3 centers in both groups of proteins share a similar coordination geometry involving two copper ions bound to three histidine residues each and coupled to each other through a bridging ligand. Thus, the study of the structural and electronic factors defining the reactivity and dioxygen binding properties in the T3 centers has represented a major issue in the understanding of their reactivity.[17]

Different spectroscopic techniques have been employed in the past two decades to study the electronic structure of copper centers in MCOs.[4,6,8,14,18−21] Studies focused on the T1 site have mostly been aimed to address the role of the axial ligand (the only variable ligand within different MCOs) in tuning the copper redox potential according to the enzyme substrate, as well as its role in providing an efficient electron transfer pathway between the reducing substrate and the TNC along a distance of *ca.* 13 Å.[1,20] The reactive TNC has proven to be much more complicated.[5,6,8,17,22−24] Several X-ray structures are available for different multicopper oxidases,[9,10,12,25−33] providing conflicting evidence regarding the presence of exogenous ligands at the TNC and its overall geometry. The structure of oxidized ascorbate oxidase[9] reveals the presence of a hydroxide bridging moiety between the two copper ions at the T3 site, and a vacant coordination position at the T2 site pointing to the center of the TNC. In several laccases (*M. albomyces* laccase, *B. subtilis* CotA),[10,34,35] a dioxygen molecule has been found, with the oxygen atoms amid the three copper ions at the cluster. This situation is complicated further by the fact that the T2 copper ion is often found with partial occupancy, or even missing in several structures.[32−34,36] Thus, the electronic description of the resting state of the TNC in MCOs is still unclear.[34]

Spectroscopic studies have been useful in elucidating the electronic structure of the TNC. These studies have included (1) analyses of the cluster in the resting state and of its interaction with small anions which inhibit the enzyme or mimic the substrate,[14,21,37−44] (2) attempts to trap reaction intermediates,[22−24] and (3) synthesis of model complexes.[5,45,46] The spectroscopic description of the TNC in the oxidized state is in agreement with the picture provided by the structure of ascorbate oxidase with two Cu^{2+} ions at the T3 site bridged by a hydroxide ligand, and a T2 copper ion with unsaturated coordination positions.[14,19]

Spectroscopic studies on the TNC, however, have been limited by the fact that the T3 center is diamagnetic in both oxidation states. In the oxidized form, $S = 0$ in the ground state since the two Cu^{2+} ions are antiferromagnetically coupled through an exogenous bridging molecule, thus precluding its analysis by EPR, ENDOR, or low-temperature MCD. These magnetic techniques are able to monitor the features of the T2 Cu^{2+} ion only, thus potentially masking magnetic interactions in the cluster.[14] NMR, instead, can be employed to interrogate T3 centers since the first excited state ($S = 1$) is paramagnetic

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and becomes populated at room temperature.[47−49] NMR of oxidized copper proteins has been largely overlooked, mostly due to the slow electron relaxation times of Cu^{2+} ion which induce extremely fast relaxation rates in nearby nuclei, rendering them undetectable. It has been shown, however, that these unfavorable electron relaxation features are restricted to T2 copper sites, since T1, T3, and Cu_A centers display faster electron relaxation rates which make them amenable to NMR studies.[47−52]

Fet3p from *Saccharomyces cerevisiae* is an MCO which oxidizes Fe^{2+} to Fe^{3+}.[8,53] Fet3p contains one T1 copper ion involved in Fe^{2+} oxidation and a TNC (Figure 1).[25] The Fet3 protein represents a unique system to employ NMR in the study of its copper sites due to its relatively small size (compared with other MCOs) and the ease of constructing site-directed mutants in the gene encoding Fet3p, *FET3*, and then producing the recombinant mutant proteins in yeast in a soluble form.[18]

Here we report a ^{1}H NMR study of the isotropically shifted signals in the vicinity of all the Cu^{2+} centers of Fet3p, which allows us to provide a detailed description of the magnetic interactions between the paramagnetic centers in the TNC, including evidence of exchange coupling between the T2 and T3 sites. By using specific mutants of the Fet3 protein we have been able to (1) assign all hyperfine-shifted signals to specific copper sites, despite the complexity of the spectrum and the unfavorable relaxation rates; (2) quantify the antiferromagnetic coupling at the T3 center in the native protein and in all the employed variants; and (3) obtain evidence of exchange coupling between the T2 and the T3 centers, thus suggesting the presence of bridging ligands involving the three copper ions at the trinuclear cluster in the resting state form of the oxidase and accounting for the unusual temperature dependence of the EPR spectra of MCOs.[21,41−44,54]

Experimental Procedures

Protein Preparation. The Fet3 proteins employed here were purified and characterized as described elsewhere.[18,55] In particular, the Cu content of all protein species was quantified by flameless atomic absorption spectrophotometry to confirm the expected copper stoichiometry. Protein samples for NMR experiments were prepared in 100 mM MES buffer at pH 6.0 in either 10% or 100% D_2O as required for each experiment and concentrated to 250−300 μL to obtain samples of *ca.* 700 μM protein.

Nuclear Magnetic Resonance Spectroscopy. NMR experiments were carried out on a Bruker Avance II spectrometer operating at 600.13 MHz (^{1}H frequency) using a triple-resonance (TXI) probehead. ^{1}H spectra were recorded with a $\pi/2$ pulse preceded by presaturation of the water signal, on a spectral window of *ca.* 100 kHz and with a total recycle time of *ca.* 220 ms. T_1 relaxation times for the hyperfine-shifted resonances were measured using the inversion−recovery method.[56] Fits of the temperature dependence of the hyperfine-shifted resonances to eq 1 were performed using the software Matlab v.7.4.0 (R2007a), The MathWorks, Inc. Only nonoverlapped signals with an anti-Curie behavior were included in the fits. Diamagnetic chemical shifts were approximated as the mean values for solvent-exchangeable and nonexchangeable protons in a His-imidazole ring (δ_{dia} = 9.5 and 7.5 ppm, respectively) taken from statistics available at the BMRB. Data analysis was carefully done following each particular resonance through the whole assayed temperature range.

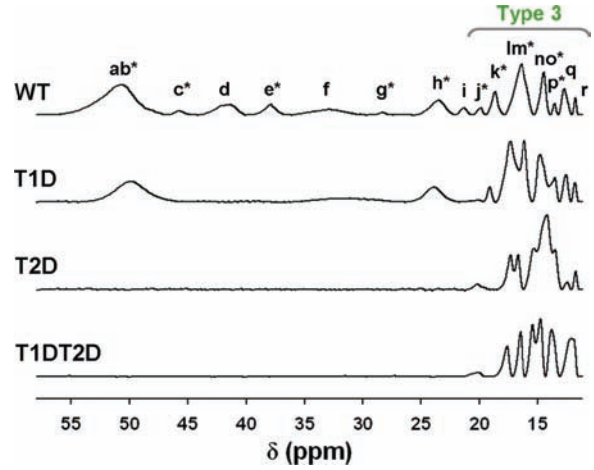

Figure 2. 600 MHz ^{1}H NMR spectra of Fet3p variants in H_2O (100 mM MES, pH 6.0) at 298 K. Signals indicated with an asterisk were absent in samples prepared in D_2O. FIDs were processed with a preexponential factor of 80 Hz.

Results and Discussion

Characterization of ^{1}H NMR Spectra of Fet3p Variants. The ^{1}H NMR spectrum of wild type Fet3p showed 18 hyperfine-shifted signals in the downfield region whereas no resonances were found upfield to the diamagnetic envelope (Figure 2). Cu^{2+} ions in T1 and T3 sites display electron relaxation times of 10^{-10} s and 10^{-11} s, respectively, making these sites accessible to NMR studies.[57] Thus, in principle, only signals corresponding to T1 and T3 ligands should be expected. Ten of the observed signals were absent in a spectrum recorded in D_2O and could then be assigned to solvent-exchangeable NH protons from His ligands (asterisks in Figure 2). Their chemical shifts fall within 55 and 11 ppm, in agreement with signals reported for copper-bound His residues from other T1[51,52,58,59] and T3 sites.[47−49] Fet3p lacks an axial ligand at the T1 site (Figure 1) since the methionine present in many cupredoxins and in several MCOs is replaced by a noncoordinating leucine residue.[25] This is consistent with the absence of an intense signal at *ca.* 28 ppm that should be expected for a Met ligand, as has been reported for *Rhus vernicifera* laccase[60] (assigned by comparison to the spectrum of Cu^{2+}-plastocyanin).[59] Resonances from the Cys

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ligand bound to the T1 Cu^{2+} ion cannot be detected directly, since the copper−sulfur covalency gives rise to a large electron spin density onto the Cys nuclei (with hyperfine coupling constants as large as 28 MHz).[52] Thus, the β-CH_2 resonances of the Cys ligand, a hallmark of T1 sites, are broadened beyond detection.[52,59] Assignment of these resonances in the small blue copper proteins has been pursued by means of a series of "blind" saturation transfer experiments in samples containing the reduced and oxidized variants in electron self-exchange.[51,52,59] These conditions cannot be attained in an MCO, thus precluding the use of this strategy. The broad nature of the observed resonances, due to both the molecular weight of the protein (∼61 kDa) and the paramagnetism, also prevented their assignment by means of ^{1}H-based NOE or 2D experiments. Note also that all detectable resonances likely correspond to imidazole protons, preventing their assignment to a particular copper site.

We thus resorted to the study of three mutants in which the metal binding capability of each mononuclear site was abolished by mutation of a key ligand. In T1D (type 1-depleted) Fet3p, the Cys484Ser replacement eliminates binding of the T1 copper ion; binding of the T2 copper ion is impaired by mutation of histidine 81 to glutamine, giving rise to the T2D (type 2-depleted) variant. T1DT2D is a double mutant combining the two previous mutations and thus possesses only the binuclear T3 center. Both single mutants bind three copper ions per molecule while T1DT2D binds only two copper ions. None of these mutants display ferroxidase activity, but the spectroscopic signatures in absorption, EPR, and EXAFS studies of the remaining metal sites are preserved upon the different mutations.[18]

We recorded the ^{1}H NMR spectra of these three variants: T1D, T2D, and T1DT2D Fet3p, which were compared to that of the wild type protein. Signals *a*, *c*, *d*, and *e* (labeling from the wild type Fet3p spectrum) were absent in the spectrum of T1D Fet3p (Figure 2), indicating that they correspond to T1 site His ligands. Their chemical shifts and line widths are in agreement with those previously reported for T1 sites.[51,52,58,59] Signals *b*, *f*, and *h* were present in the spectrum of T1D Fet3p (Figures 2 and S1) but absent in the spectrum of T2D Fet3p, suggesting that they may correspond to T2 copper ligands. These three resonances resemble those present in the spectrum of T1Hg *Rhus* laccase, which were tentatively attributed to the T3 site.[60] The present data, however, allow us to unequivocally assign them as signals from the T2 site. Surprisingly, the spectrum of T2D Fet3p also lacked the T1 resonances despite the fact that the optical signature of the T1 Cu^{2+} center[18] at 16 600 cm^{-1} was unaltered in this sample. Consistently, the spectrum of the T1DT2D mutant resembled that of T2D Fet3p (Figure S2). One possible explanation for the absence of T1 signals in the T2D Fet3p NMR spectrum is to assume an unfavorable chemical exchange regime. A more flexible conformation of His416 when the T2 Cu^{2+} ion is not bound could be transmitted to the T1 site through His413 (Figure 1). This proposal is supported by an electrochemical study on *Rhus* laccase, which shows that removal of the T2 metal ion induces a 10-fold increase in the reduction entropy of the T1 site.[60]

A group of sharper signals at $\delta < 21$ ppm was present in all variants; these, then, can be unequivocally attributed to His ligands of the binuclear T3 center, which is the only unperturbed metal site in all of the variants. These resonances show slower nuclear relaxation rates than those arising from the T1 or T2 site, as expected from the faster relaxation reported for only-T3 systems, such as in tyrosinase.[47,48]

Temperature Dependence of T3 Resonances. ^{1}H NMR spectra of all Fet3p variants were recorded at different temperatures in the 278 to 304 K range. Resonances from type 1 and type 2 copper sites followed a Curie-like behavior (Figure S3). All signals attributed to T3 ligands showed temperature behavior deviating from Curie's law in all variants (Figure 3). As shown by Canters and co-workers for tyrosinase, this behavior can be accounted for by assuming a diamagnetic ground state and a temperature-accessible $S = 1$ excited state located at an energy of $-2J$ above the ground state.[47,48] The contact chemical shifts of the isotropically shifted resonances at different temperatures can be related to the J value by the following equation:[48]

$$\delta_{observed} = \delta_{diamagnetic} + \frac{A}{T}\frac{g_e\mu_B}{\hbar\gamma k_B}\left[\frac{\exp(2J/k_BT)}{1 + 3\exp(2J/k_BT)}\right]\cdot 10^6 \text{ ppm} \quad (1)$$

This model is valid for an isolated T3 site, such as the one present in the T2D and T1DT2D variants. Analysis of the temperature dependence data of these mutants yielded a $-2J$ value of 660 cm^{-1} for both of them (Figure 3). This value is in agreement with the lower limit of 400 cm^{-1} obtained by magnetic susceptibility measurements on T2D *Rhus* laccase[14] and a value of *ca.* 500 cm^{-1} estimated from DFT calculations in both native and T2D *Rhus* laccase.[19] The value obtained here for Fet3p is sizably larger than that determined by NMR for the isolated type 3 site in tyrosinase ($-2J \approx 100$ cm^{-1}).[47] The larger exchange coupling constant is consistent with the smaller hyperfine shifts reported here compared to those observed for tyrosinase (which span up to 50 ppm), resulting from a less populated $S = 1$ level in MCOs.

The NMR spectrum of *Rhus* laccase[60] does not reveal the presence of relatively sharp resonances (which could be attributed to His ligands from the T3 site) with chemical shifts higher than 25 ppm, thus suggesting a similar exchange coupling at the T3 center in both MCOs. Tentatively, the same situation holds for the small laccase SLAC from *S. coelicolor*. Although values for the exchange coupling constants are not available for SLAC, the appearance of four signals with anti-Curie behavior and chemical shift values smaller than 25 ppm suggest a similar situation for this protein.[49]

The temperature dependence of T3 signals in wt and T1D Fet3p variants, both containing a TNC, are qualitatively similar to those of the T2D and T1DT2D mutants, suggesting that the T2 Cu has only a minor influence on the magnetic properties of the T3 site. Analysis of the temperature behavior of these variants with the same model (*i.e.*, assuming an isolated T3) resulted in similar $-2J$ values (540 cm^{-1} for wt and 580 cm^{-1} for T1D Fet3p). These results suggest that the T2 site does not significantly perturb the coupling in the T3 center in the resting state, in agreement with DFT calculations on the related oxidase *Rhus* laccase.[19]

We can then conclude that (1) T3 sites in MCOs display sizably larger exchange coupling constants than T3 sites in tyrosinase and (2) this situation is independent of the T2 copper ion.

Analysis of the T2−T3 Coupling. The temperature dependence analysis of the T3 resonances indicates that the presence of the T2 Cu^{2+} ion does not significantly affect the exchange coupling between the two T3 Cu^{2+} ions, in agreement with DFT

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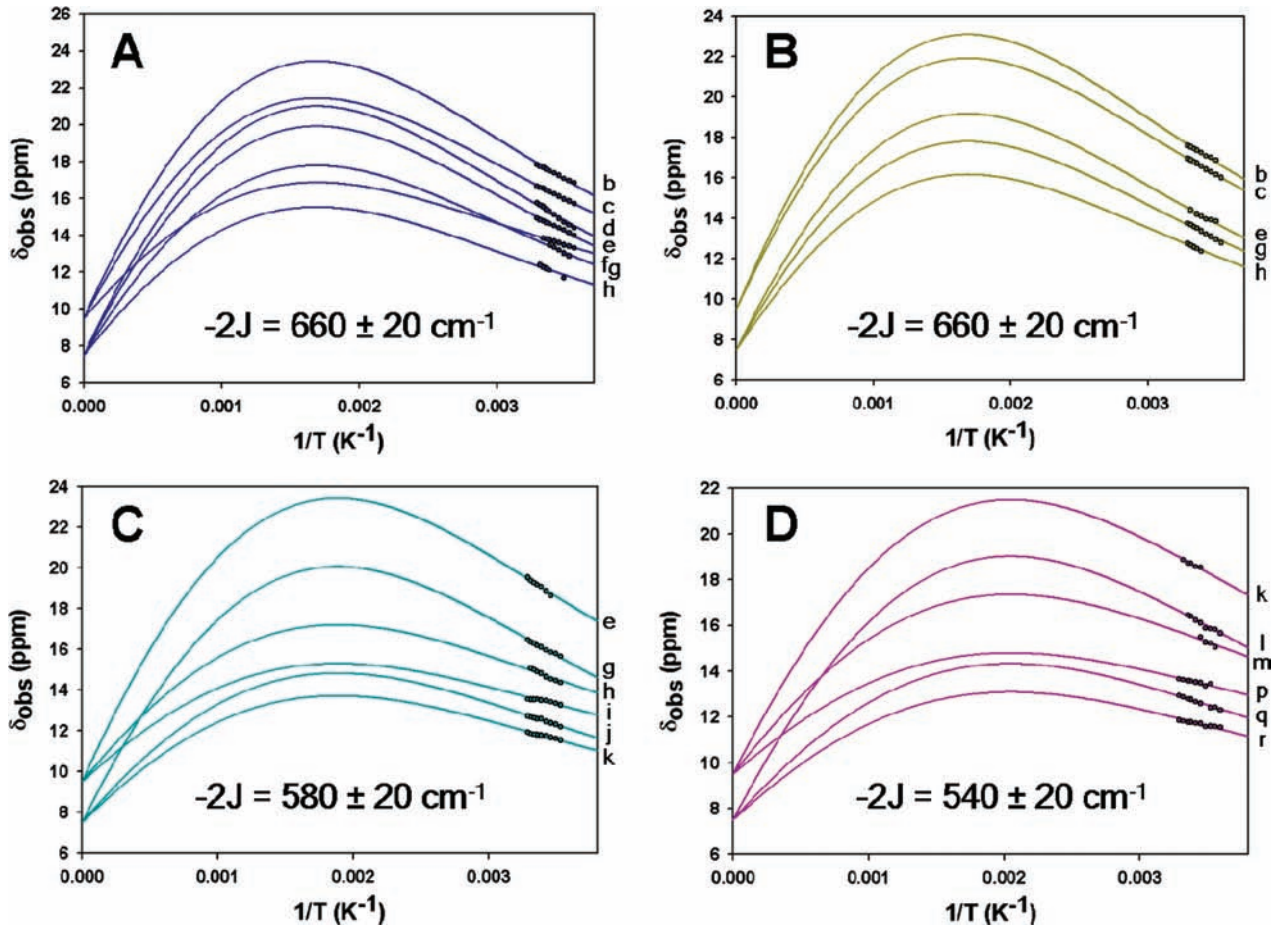

Figure 3. Fits of the temperature dependence data to eq 1 for T3 resonances in T1DT2D (A), T2D (B), T1D (C), and wild type (D) Fet3p variants. Indicated errors correspond to least-squares errors in the fitting. Labels for the curves are indicated on the right of each plot and refer to resonance labeling shown in Figure S2 for each Fet3p variant.

calculations.[19] Despite this conclusion, the direct detection of resonances from ligands bound to the T2 Cu^{2+} ion (signals *b*, *f*, and *h* from wt Fet3p spectrum in Figure 2) is not consistent with the presence of a magnetically isolated T2 site, whose typical τ_s value (*ca.* 10^{-9} s) is expected to broaden the NMR resonances of the metal ligands beyond the detection limit.[61]

The spectral features of the resonances here detected suggest a τ_s of *ca.* 10^{-10} s for the T2 site, which can be achieved by coupling of this Cu^{2+} ion to a fast relaxing center, such as the T3 site.[62] This coupling can be dipolar or exchange in origin. Assumption of dipolar coupling (eq 2) to the metal ions at the T3 site, located at *ca.* 3.7 Å from the T2 center,[9] cannot account for this shortening in τ_s, even assuming a fully populated $S = 1$ for the type 3 center.[62] Instead, very small $|J|$ values (calculated using eq 3)[62] can give rise to significant changes in τ_s values. For example, in the case of $Cu^{2+}-Co^{2+}$ superoxide dismutase, a J value of 16.5 cm^{-1}, mediated through a common His ligand, is able to elicit electron relaxation rates in a T2 Cu^{2+} ion which match those of the fast relaxing high-spin Co^{2+}, giving rise to detectable NMR resonances.[63] In this case, by assuming a population of 5% of the $S = 1$ state and a range of possible electron relaxation times for the isolated T2 and T3 centers available from the literature, we have estimated that a $|J|$ value between 1 and 4 cm^{-1} is able to account for the observed features (see Table S1).

$$\tau_{SM1}^{-1}(J) = \tau_{SM1}^{-1}(0) + \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{g_e^4 \mu_B^4 S_2(S_2+1)}{\hbar^2 r^6} \times \left(\frac{\tau_{SM2}}{1+(\omega_{SM1}-\omega_{SM2})^2\tau_{SM2}^2} + \frac{3\tau_{SM2}}{1+\omega_{SM1}^2\tau_{SM2}^2} + \frac{6\tau_{SM2}}{1+(\omega_{SM1}+\omega_{SM2})^2\tau_{SM2}^2}\right) \quad (2)$$

$$\tau_{SM1}^{-1}(J) = \tau_{SM1}^{-1}(0) + \frac{2}{3}\left(\frac{J^2}{\hbar^2}\right) S_2(S_2+1) \times \left(\frac{\tau_{SM2}}{1+(\omega_{SM1}-\omega_{SM2})^2\tau_{SM2}^2}\right) \quad (3)$$

Such a small exchange coupling is not expected to perturb the T3 site, supporting the fact that the temperature dependence of the NMR shifts can be accurately described by a singlet−triplet splitting (as discussed above). This picture is also consistent with the finding of similar NMR signals in *Rhus* laccase,[60] suggesting that this small exchange coupling could be a general feature of the resting state of multicopper oxidases. The NMR

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spectrum of *S. coelicolor* laccase SLAC shows a large number of hyperfine-shifted signals that might well correspond to a T1 and a T2 site.[49]

The small exchange coupling between the T2 Cu^{2+} ion and the T3 site (either Cu3α or Cu3β, or both) is relevant to the electronic structure of the resting TNC in MCOs and allows us to account for the temperature dependence of the EPR spectrum of the T2 Cu^{2+} center. Morpurgo and co-workers[44] originally reported changes in the EPR features of this center between room temperature and 77 K, as later studied by McMillin[21,41,54] and Sakurai.[42,43] At room temperature, the EPR parameters reflected a tetrahedral distortion of the metal site and (surprisingly) a 70% reduction in the intensity of the EPR signal. These results were accounted for by assuming the existence of different enzyme conformational states[21,41−44,54] or the population of a low-lying electronic excited state at room temperature.[14] The enhanced electron relaxation herein reported for the T2 Cu^{2+} ion can explain the loss of EPR signal at room temperature.

This coupling within the TNC indicates the presence of a bridging ligand between the T3 and the T2 metal ions in the resting state of MCOs. Crystal structures of different laccases have disclosed the presence of a dioxygen molecule bound to the TNC, making direct contacts not only with the T3 copper ions but also with the T2 ion.[10,34] Our results are consistent with this observation. An alternative explanation is related to the finding that both T2 and T3 sites have open coordination positions oriented toward the center of the cluster,[19] which could allow binding of a solvent molecule providing this coupling. Thus, the present NMR data fill a gap in the description of the electronic structure of the trinuclear clusters in multicopper oxidases. We therefore envisage the use of NMR as highly promising to complement other techniques in the analysis of the electronic structure of the metal sites in multicopper oxidases.

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Supporting Information Available: Characterization of the ^{1}H NMR signals in all Fet3p variants, temperature dependence data for resonances from type 1 and type 2 copper sites, and *J* values obtained from eq 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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