Homogeneous catalytic $\text{O}_2$ reduction to water by a cytochrome $c$ oxidase model with trapping of intermediates and mechanistic insights

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An efficient and selective four-electron plus four-proton (4e$^-$/4H$^+$) reduction of $\text{O}_2$ to water by decamethylferrocene and trifluoroacetic acid can be catalyzed by a synthetic analog of the heme $a_2$/Cu$_b$ site in cytochrome $c$ oxidase ($6\text{LFeCu}$) or its Cu-free version ($6\text{LFe}$) in acetone. A detailed mechanistic-kinetic study on the homogeneous catalytic system reveals spectroscopically detectable intermediates and that the rate-determining step changes from the $\text{O}_2$-binding process at 25 °C room temperature (RT) to the O-O bond cleavage of a newly observed Fe$^{III}$-OOH species at lower temperature (~60 °C). At RT, the rate of $\text{O}_2$-binding to $6\text{LFe}$ is significantly faster than that for $6\text{LFeCu}$, whereas the rates of the O-O bond cleavage of the Fe$^{III}$-OOH species observed (~60 °C) with either the $6\text{LFeCu}$ or $6\text{LFe}$ catalyst are nearly the same. Thus, the role of the Cu ion is to assist the heme and lead to faster O-O bond cleavage at RT. However, the proximate Cu ion has no effect on the O-O bond cleavage of the Fe$^{III}$-OOH species at low temperature.

heme/copper | dioxygen reduction | ferric hydroperoxo | kinetic mechanism | enzyme model

The heme/copper (heme $a_2$/Cu$_b$) heterodinuclear center in cytochrome $c$ oxidases (CcO) (Fig. L4) has attracted much interest, because this is the site where the four-electron and four-proton reduction of oxygen takes place as the final stage of the respiration chain (Eq. 1).

$$\begin{align*} 
\text{O}_2 + 4e^- + 4\text{H}^+ & \rightarrow 2\text{H}_2\text{O} + 8\text{H}^+ \\
& \text{(from cyt-c reduced) + 8 H}^+ \text{(from inside membrane)} \\
& \rightarrow 2\text{H}_2\text{O} + 4\text{H}^+ \text{(membrane translocated) + 4 cyt-c oxidized} \\
\end{align*}$$

[1]

This exergonic process, occurring without leakage of harmful partially reduced oxygen species, is coupled to the translocation of four additional protons across the membrane, generating a pH gradient and membrane potential which is harnessed through the subsequent synthesis of ATP (1–4).

In addition to protein crystallography, mechanistic enzymology, site-directed mutagenesis, and theoretical calculations, biomimetic inorganic modeling of the CcO active site has been employed. The goal is to provide insights and elucidate mechanisms of the four-electron reduction of $\text{O}_2$ by coordination complexes including heme-Cu assemblies, as may be relevant to biological systems including CcO but also of technological significance such as in fuel cell chemistry (5–7).

A number of heme $a_2$/Cu$_b$ synthetic analogues have been developed to mimic the coordination environment of the heme $a_2$/Cu$_b$ bimetallic center in CcO (8–11). The functionality of these analogues has been investigated under two main complementary approaches. One focuses on the generation and characterization of stable $\text{O}_2$-adducts and derived species in heme-copper synthetic assemblies (8, 9). This stoichiometric approach represents an efficient tool to probe the properties and plausibility of the intermediates relevant to the enzyme catalytic cycle and/or O-O reductive cleavage chemistry, the latter of critical importance in chemical and biochemical utilization of molecular oxygen. The second approach, based on electrochemical functional modeling, examines the capability of synthetic models to perform the catalytic four-electron reduction of $\text{O}_2$ (10, 11). However, the solid supported state employed for such studies has precluded any spectroscopic monitoring or intermediates detection. This problem is a major obstacle for the development of kinetics and mechanistic studies in synthetic models or to answer important mechanistic questions such as the role of the Cu$_b$ in the four-electron reduction of $\text{O}_2$.

As an alternative to the limitations of the two approaches described above, we report herein an efficient 4e$^-$/4H$^+$ catalytic reduction of $\text{O}_2$ to water, catalyzed by a heme/Cu functional model of CcO ($6\text{LFeCu}$, Fig. 1A) and its Cu-free version ($6\text{LFe}$, Fig. 1C). As described below, this homogeneous catalytic system has allowed us to develop a detailed kinetic description supported by spectroscopic detection of reactive intermediates overall providing unique mechanistic insights into the O-O reductive cleavage process (12).

We previously reported that the reduced form of $[\text{Fe}^{III}\text{Cu}^{I}]^+ \cdot \text{B(C}_6\text{F}_5)_2$ reacts with $\text{O}_2$ to form a low-temperature stable $\text{Fe}^{III}_{\mu-\text{peroxo-Cu}^{II}}$ ($[\text{Fe}^{III}_{\mu-\text{peroxo-Cu}^{II}}(\text{O}_2^{2-})^\cdot \text{Cu}^{II}]^+) \cdot \text{B(C}_6\text{F}_5)_2$ complex ($\Delta \nu_{\text{O-O}} = 808$ cm$^{-1}$, $\Delta \nu_{\text{O-O}} = -23$ cm$^{-1}$), deduced to be very similar to a crystallographically characterized $\text{Fe}^{III}_{\mu-\text{peroxo}-\text{Cu}^{II}}$ complex reported by Naruta and coworkers (13). The $[\text{LFe}^{III}_{\mu-\text{peroxo}-\text{Cu}^{II}}]^+$ complex thermally...

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Fig. 1. (A) X-ray structures of the fully reduced bimetallic heme $a_2$/Cu$_b$ center in CcO from bovine heart (Fe$^{III}$–Cu$^{II}$ = 5.19 Å) (3) [figure adapted from (8)]. (B) heme/Cu synthetic model for CcO ($6\text{LFeCu}$). (C) Cu-free version of synthetic model for CcO ($6\text{LFe}$).

Catalytic O2-Reduction at −60 °C. When the time course for catalytic reaction Eq. 2 is followed by UV-visible spectroscopy at −60 °C (Fig. 2A), two main changes occur. There is a rise in the absorbance at 780 nm due to the formation of ferrocenium cation (Fe3+) as product formed following electron-donation by Fc+ (1.0 mM) catalyzed by 6LFeCu (1.0 μM) in the presence of TFA (1.0 mM) in air-saturated acetone ([O2] = 2.2 mM) at −60 °C. The insert shows the time profile of the absorbance at 780 nm relative to Fe3+ formation. Note: a tiny amount of Fe3+ is formed (see the nonzero absorbance) during the mixing time following combining catalyst and ferrocene solutions (in order to obtain a homogeneous solution) and before the first spectrum is recorded. Thus, for practical reasons, this first spectrum corresponds to time = 0. (B) Plots of kobs vs. [cat] for 6LFeCu and 6LFe at −60 °C. (C) Plots of kobs vs. [Fc+] for 6LFeCu and 6LFe at −60 °C. (D) Plots of kobs vs. [TFA] for 6LFeCu and 6LFe at −60 °C. (E) Plots of kobs vs. [O2] for 6LFeCu and 6LFe at −60 °C.

The stoichiometry of the overall reaction was confirmed by the observation that exactly four equiv of Fe3+ form per mole of O2 reacted (i.e., under limiting [O2]i), H2O2 as a product formed via the partial two-electron reduction of O2 was ruled out by iodometric titration experiments (Fig. S6); no hydrogen peroxide was detected. In addition, it was confirmed that O2 reduction by Fe3+ without catalysts (6LFeCu or 6LFe) is negligible under the same conditions (Fig. S6).

To help to examine the role of the Cu in this system, a Cu-free version (6LFe) (15) was also subjected to the same catalytic reaction conditions as 6LFeCu. Even in the absence of Cu the reaction proceeds through the 4e− process (Fig. S7). This result is in accordance with earlier electrochemical studies in which it was found that only the iron porphyrinate itself is essential for O2 four-electron reduction (17–20). In the case of 6LFeII as well, the kinetics reveal that the Fe3+−OOH complex represents the catalytic cycle steady-state species (Fig. S7A).

Further kinetic insights demonstrate the reactions being studied are zero-order with respect to Fc+ formation for both 6LFeCu (Fig. 2A, inset) and 6LFe (Fig. S7B) as catalysts. The derived rate constants increase linearly with an increase in catalyst concentrations (Fig. 2B) and also remain constant with a change in the concentration of Fc+ (Fig. 2C). Further, and to our surprise, the observed zero-order rate constants (kobs) with 6LFeCu at −60 °C are exactly the same as those with the Cu-free complex 6LFe (Fig. 2B). Also, the rate of the reaction remains constant with an increase in [TFA] or [O2] (Fig. 2 D and E).

These data demonstrate that the rate of O2-reduction is not affected by the concentrations of O2, TFA, or Fc+. Thus, the rate-determining step of the catalytic reaction at low temperature is O−O bond cleavage in the steady-state Fe3+−OOH species and this is followed by rapid electron transfer to complete the 4e− reduction of O2. The same kobs value observed for 6LFeCu and

Results and Discussion

Catalytic O2-Reduction at −60 °C. When the time course for catalytic reaction Eq. 2 is followed by UV-visible spectroscopy at −60 °C (Fig. 2A), two main changes occur. There is a rise in the absorbance at 780 nm due to the formation of ferrocenium cation (Fe3+) as product formed following electron-donation by Fc+ (1.0 mM). The strong absorbance at 415 nm corresponds to the Soret band of the heme in its steady-state form in the catalytic cycle. This reaction intermediate has been determined to be a unique 6LFeIII-hydroperoxo Cu (6LFeIII(O2−)-CuIII) species (415, 538 nm); 6LFeIII−OOH CuHII(2+) could be independently generated at low temperature (−80 °C) by the addition of an excess of TFA to the previously described peroxo complex 6LFeIII−O2−(CuHII)+ (418, 540, 558 nm) (Fig. S1). The hydroperoxide formulation is further supported by electrospore ionization mass spectrometry (ESI-MS) data and EPR spectroscopic monitoring of its formation (Figs. S2 and S3); the addition of an excess TFA to the EPR-silent 6LFeIII−O2−(CuHII)+ complex resulted in the display of a characteristic signal (g = 6.08) of a five coordinate high-spin heme and typical signal for the CuII moiety (A1 = 142 × 10−4 cm−1, A2 = 12 × 10−4 cm−1, g1 = 2.26 and g1 = 2.05)9. Hydroperoxo complex O−O reductive cleavage by 2Fc+/3H2O (to two waters) leads to {6LFeIII(CuII)}+ which undergoes further two-electron (2 Fc+) reduction to the fully reduced form {6LFeIII(CuI)}+; these observations also strongly support the (6LFeIII−OOH Cu) steady-state species formulation (Figs. S4, S5).

In 6LFeIII−O2−(CuHII)+ the high-spin FeII (S, S = 5/2) system is strongly antiferromagnetically coupled to the CuII ion (S, S = 1/2) through the peroxo bridge, giving an overall S = 2 spin system and an EPR-silent spectrum. The addition of the excess of TFA causes the formation of the FeII−OOH CuIII in which the Fe and the Cu are no longer antiferromagnetically coupled and both the high-spin FeII and the CuII ion centers independently display an EPR signal (Fig. S3).

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\(^6\)LFe suggests that the Cu is not bound to the \(\text{Fe}^{III}\)-OOH moiety in \((\text{FC})\text{LFe}^{III}\)-OH Cu).

**Catalytic \(O_2\)-Reduction at 25 °C.** The spectral changes observed for the catalytic reaction (Eq. 2) at RT are quite different from those observed at \(-60\) °C. At RT, the Soret band corresponding to the steady-state is observed at 422 nm (Fig. 3A), and this can be assigned to the reduced heme (Fe(II)) (16). Further confirmation comes from spectral correlation with the species generated in the absence of \(O_2\) using Fe(II) in the presence of TfA (Figs. S5 and S8).

In the presence of excess \(O_2\), the reaction kinetics are zero-order in Fe(II) formation (Fig. 3A, inset), while the zero-order rate constant increases linearly with an increase in the catalyst concentration (Fig. 3B, Fig. S9), as expected. More interestingly, the rate constant also increases with an increase in the \(O_2\) concentration (Figs. 3E, Fig. S10), but remains constant with increases in the acid or Fe(II) concentrations (Fig. 3C). On the basis of these observations, and knowing that the reduced heme (Fe(0)) is the steady-state species observed for the catalytic cycle (vide supra), we can conclude that the rate-determining step at 25 °C is the \(O_2\)-binding to the \(\text{Fe}^{III}\) catalyst complex.

As well, our kinetics investigations demonstrate that for the Cu-free catalyst, the reduced heme (Fe(0)) is the species found to be in a steady-state. Thus, \(O_2\)-binding is rate-determining. However, the rate constant determined (as measured by Fe(II) formation) is approximately two times less than that observed with \((\text{FC})\text{LFe}^{III}\) (Fig. 3B). Consequently, the efficiency of the catalyst, as represented by the turnover frequency, is greater for \((\text{FC})\text{LFe}^{III}\) (TOF = 41 s\(^{-1}\)) than that for \(\text{Fe}^{III}\) (TOF = 24 s\(^{-1}\)). This result suggests that the role of the Cu, at ambient temperature, in this biomimetic catalytic system, is to assist the heme and lead to faster \(O_2\)-binding during the catalytic cycle.

We can compare the results here with this heme-Cu CoO model compound to systems studied under similar conditions, in homogeneous solutions with ferrocene derivative reductant sources. \(\text{Fe}^{III}\)CoO offers a significantly higher efficiency (2.2 × 10\(^6\) s\(^{-1}\), 298 K, slope in Fig. 4C) than that observed for previously described cofacial dicobalt porphyrins (320 s\(^{-1}\)) or with a mononuclear copper complex (17 s\(^{-1}\)) (21–23).

Another role for Cu, as an electron storage and delivery site, was previously proposed by Collman and coworkers based on electrochemical studies (24). This difference may result from the fact that, in the electrochemical approach, the electron flow to the catalyst is controlled, in contrast with our solution homogeneous system. Also, an electrode material supported catalyst may possess a structure modified from that observed in solution (25).

**Variable Temperature (VT) Studies.** As deduced from Fig. 4A, VT studies provide a clearer picture of the change in the rate-determining step for the catalytic 4e\(^-\)/4H\(^+\) reduction of \(O_2\) by Fe(II) with TfA. At \(T < -5\) °C, the O-O bond cleavage of \(\text{Fe}^{III}\)-OH is rate-determining.

Because the Cu is not involved here, the zero-order rates \((k_{obs})\) for \(\text{Fe}^{III}\) are nearly the same as those of \((\text{FC})\text{LFe}^{III}\) (Fig. 4A and C). Between \(-60\) and \(-5\) °C (Fig. 4C), the activation energy determined for \((\text{FC})\text{LFe}^{III}\) (9.4 ± 0.1 kcal mol\(^{-1}\)) and \(\text{Fe}^{III}\) (9.9 ± 0.2 kcal mol\(^{-1}\)) are essentially the same.

At higher temperature (\(T > -5\) °C), however, the \(O_2\)-binding to the reduced species \((\text{FC})\text{LFe}^{III}\) or \(\text{Fe}^{III}\) becomes rate-determining. The difference in the \(k_{obs}\) value between \(\text{Fe}^{III}\) and \(\text{Fe}^{III}\) becomes larger with an increase in temperature and at physiological temperature (37 °C) the \(k_{obs}\) value for \(\text{Fe}^{III}\) becomes seven times larger than that of \(\text{Fe}^{III}\).

Fig. 4B shows the change in Soret band as a function of reaction temperature. This phenomenon clearly represents a change in the identity of the steady-state complex for the low and higher temperature processes: The 415–417 nm absorption representing the \(\text{Fe}^{III}\)-OH complex in steady-state, shifts to 422–424 nm, identified as the reduced (Fe(II)) heme complex. Notably, and showing the consistency of our findings and conclusions, the boundary temperature is about \(-5\) °C for the different analyses represented by Fig. 4A–C.

Kinetic analyses and Arrhenius plots (Fig. 4C) show that the catalytic \(O_2\)-reduction using \(\text{Fe}^{III}\) gives an activation energy of 4.2 ± 0.1 kcal mol\(^{-1}\) between \(-5\) and 35 °C. However, with the \(\text{Fe}^{III}\) catalyst, \(k_{obs}\) values for \(O_2\)-binding decrease with increasing temperature affording an apparent negative activation energy of \(-3.0 \pm 0.1\) kcal mol\(^{-1}\). Although the rate of Fe(II) formation obeys approximately zero-order kinetics, a more careful examination of the data for \(\text{Fe}^{III}\) reveals that the apparent zero-order rate constant increases with increasing Fe(II) concentration (Fig. 3C, Fig. S11B). By contrast, for \(\text{Fe}^{III}\), the zero-order rate constant remains the same under such conditions (Fig. 4C, Fig. S11A). These observations indicate that the binding of
plex thus generated undergoes a fast protonation to form $\text{LFeCu}^+$. The back reaction (i.e., releasing $\text{O}_2$) starts via a fast reduction of the heme and then the Cu to generate the reduced complex $\text{LFe}^-$. The $\text{O}_2$-binding is rate-determining at RT. The $\text{O}_2$-binding is slower than that observed for $\text{LFe}^{\text{II}}\text{Cu}^{\text{II}}$. Once an $\text{O}_2$-adduct forms [formally an $\text{Fe}^{\text{III}}$, superoxo species (418, 539 nm) (16)], subsequent electron transfer and protonation leads to $\text{Fe}^{\text{III}}\text{OOH}$, in steady-state at low temperature. Thus, the important role of Cu is to facilitate the $\text{O}_2$-binding to $\text{LFe}^{\text{III}}$, directly increasing the rate of this reaction and stabilizing the resulting $\text{LFe}^{\text{III}}(\text{O}_2^-\text{Cu}^{\text{II}})$ complex at ambient temperature. As seen for the $\text{LFe}^{\text{III}}\text{Cu}$ catalyst, the rate-determining step changes at low temperature, from $\text{O}_2$-binding to O-O bond cleavage in the $\text{Fe}^{\text{III}}\text{OOH}$ complex. Cu does not influence this latter step.

In fact, this conclusion about the role of Cu agrees with other notable findings: (i) $\text{O}_2$-binding to copper complexes can occur at near diffusion-controlled rates (26, 27), (ii) $\text{CcO}$ enzyme studies in fact implicate $\text{Cu}_{\text{II}}$ as the entry point for $\text{O}_2$ during catalysis (28–30).

**Conclusion**

In summary, we have here described a selective and efficient (turnovers >1,000) four-electron reduction of $\text{O}_2$ to water by $\text{Fc}^+$ in the presence of TFA in acetone by: (A) the heme/Cu bimetallic center model of CrO ($\text{LFeCu}$), (B) the Cu-free version ($\text{LFe}$). In fact, it has not yet been established as to whether the side of the heme the $\text{O}_2$-binding occurs.

Protons are needed to complete the $4e^-/4H^+$ reduction of $\text{O}_2$ to water and regenerate the $\text{[LFe}^{\text{III}}\text{Cu}]^+$ catalyst.

In the case of $\text{LFe}$, the Cu-free catalyst version, the cycle (Fig. 5B) also starts via fast electron transfer from $\text{Fc}^+$ to the heme, forming $\text{LFe}^{\text{II}}$. In the absence of the Cu, however, as discussed, the subsequent $\text{O}_2$-binding is slower than that observed for $\text{LFe}^{\text{III}}\text{Cu}^{\text{II}}$. Once an $\text{O}_2$-adduct forms [formally an $\text{Fe}^{\text{III}}$, superoxo species (418, 539 nm) (16)], subsequent electron transfer and protonation leads to $\text{Fe}^{\text{III}}\text{OOH}$, in steady-state at low temperature. Thus, the important role of Cu is to facilitate the $\text{O}_2$-binding to $\text{LFe}^{\text{III}}$, directly increasing the rate of this reaction and stabilizing the resulting $\text{LFe}^{\text{III}}(\text{O}_2^-\text{Cu}^{\text{II}})$ complex at ambient temperature. As seen for the $\text{LFe}^{\text{III}}\text{Cu}$ catalyst, the rate-determining step changes at low temperature, from $\text{O}_2$-binding to O-O bond cleavage in the $\text{Fe}^{\text{III}}\text{OOH}$ complex. Cu does not influence this latter step.

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**Conclusion**

In summary, we have here described a selective and efficient (turnovers >1,000) four-electron reduction of $\text{O}_2$ to water (without formation of $\text{H}_2\text{O}_2$) catalyzed by our CrO active site analogue ($\text{LFeCu}$). Unprecedented direct observation of different reactive intermediates taking part in the catalytic cycle [i.e., $\text{LFe}^{\text{III}}\text{Cu}^{\text{II}}$, $\text{LFe}^{\text{III}}\text{OOH}$ (Cu)], depending on the temperature of the system, combined with detailed kinetic studies of different steps of the catalytic reaction when comparing $\text{LFeCu}$ and its Cu-free version allowed us to obtain unique mechanistic insights. Important conclusions that may relate to CrO chemistry are that in our model system the role for the Cu is to enhance the $\text{O}_2$-binding and that it needs not contribute directly to the O-O cleavage process. As no model system can prove a mechanistic model for an enzyme, our chemistry provides that further systematic variations in the architecture or nature of the synthetic heme-copper catalyst [such as changes in the heme type, Cu-ligand denticity, N-donor type, neighboring groups which can H-bond or alter the local dielectric, or $\text{H}^+\ (\text{H}^+\ + e^-)$ donors] can and will...
lead to further insights into $O_2$ reductive activation relevant to CoO or fuel cell chemistry.

Materials and Methods

Materials. Grade quality solvents and chemicals were obtained commercially and used without further purification unless otherwise noted. Decamethylferrocene (Fc+) (99%) was purchased from STREM, USA, TFA (99%) from Sigma Aldrich and NaI (99.5%) from Wako, Japan. Acetone was purchased from Wako, Japan and used whether without further purification for non-air-sensitive experiment or dried and distilled under argon then deoxygenated by bubbling with argon for 30–45 min for air-sensitive experiment. Preparation and handling of air-sensitive compounds were performed under MBraun UNilab inert atmosphere (<1 ppm O$_2$, <1 ppm H$_2$O) glove box filled with nitrogen. The complexes [FcFe(CN)$_3$]$^-$ and [FeFe] were prepared according to the literature procedure (14, 15).

UV-Vis Spectroscopy Measurements. Hewlett Packard 8453 diode array spectrophotometer with a quartz cuvette (path length = 10 mm) was used to examine the spectral change in the UV-visible. This instrument was coupled to Unisoku thermostated cell holder for low-temperature experiments. In a typical catalytic reaction, the quartz cuvette is loaded with 3 mL of 1:1 Fe$^3+$TFA (1 mM) in air-saturated solution of acetone. Then 30 mL of 0.1 mM solution of the catalyst in acetone is injected into the cuvette under vigorous stirring. The catalytic reaction is monitored by the increase in the absorbance at 780 nm corresponding to the formation of the ferroocene cation (Fc$_2^+$).

The limiting concentration of $O_2$ in an acetone solution was prepared by a mixed gas flow of $O_2$ and $N_2$. The mixed gas was controlled by using a gas mixer (Kofloc GB-3C, KOJIMA Instrument Inc.), which can mix two or more gases at a certain pressure and flow rate.

stopped-flow measurements. Stopped-flow measurements were performed on a UNISOKU RSP-601 stopped-flow spectrophotometer with an MOS-type high selective photodiode array at various temperatures (248 K–298 K) using a Unisoku thermostated cell holder designed for low-temperature experiments. In a typical reaction, two reactant solutions for stopped-flow mixing were prepared. One is solution containing TFA and the catalyst in acetone, the other is solution Fe$_2^{3+}$ in acetone. Rates of $O_2$ reduction reactions were determined by monitoring the appearance of the absorption band at 780 nm due to the formation of Fc$_2^+$.

ESI-MS Measurements. The detailed information about ESI-MS is provided in SI Text.

EPR Measurements. To a reaction solution of [Fe$^{3+}$Fe($O_2$)$_3$]$^{2-}$ (Fig. S3) or [Fe$^{3+}$Fe($O_2$)$_3$]$^{2+}$ (1.0 mM) in acetone solution, 10 equiv of TFA (10 mM) was added at RT. The resulting solution in the quartz ESR tube (3.0 mm i.d.) was frozen in liquid nitrogen. EPR spectrum recorded at 77 K was taken on a JEOL X-band spectrometer (JE-RE1XE) under nonsaturating microwave power conditions (1.0 mW) operating at 9.2025 GHz (Fig. S14) or a Bruker EMX spectrometer operating at X-band using microwave frequencies around 9.5 GHz (Fig. S3). The magnitude of the modulation was chosen to optimize the resolution and the signal to noise ratio (S/N) of the observed spectrum (modulation width, 20 G; modulation frequency, 100 kHz).

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