Oxygen Activation by the Noncoupled Binuclear Copper Site in Peptidylglycine α-Hydroxylating Monooxygenase. Spectroscopic Definition of the Resting Sites and the Putative Cu$^{II}_{M}$−OOH Intermediate†

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ABSTRACT: Spectroscopic methods, density functional calculations, and ligand field analyses are combined to define the geometric models and electronic structure descriptions of the Cu$_M$ and Cu$_H$ sites in the oxidized form of the noncoupled binuclear copper protein peptidylglycine α-hydroxylating monooxygenase (PHM). The Cu$^{II}_{M}$ site has a square pyramidal geometry with a long axial Cu–methionine bond and two histidines, H$_2$O, and OH$^-$ as equatorial ligands. The Cu$_H$ site has a slightly $D_{2d}$ distorted square planar geometry with three histidines and H$_2$O ligands. The structurally inequivalent Cu$_M$ and Cu$_H$ sites do not exhibit measurable differences in optical and electron paramagnetic resonance (EPR) spectra, which result from their similar ligand field transition energies and ground-state Cu covalencies. The additional axial methionine ligand interaction and associated square pyramidal distortion of the Cu$_M$ site have the opposite effect of the strong equatorial OH$^-$ donor ligand on the Cu$^{II}_{M}$ site leading to similar ligand field transition energies for both sites. The small molecule NO$_2^-$ binds in different coordination modes to the Cu$_M$ and Cu$_H$ site because of differences in their exchangeable coordination positions resulting in these Cu$^{II}$ sites being spectroscopically distinguishable. Azide binding to PHM is used as a spectroscopic and electronic structure analogue to OOH$^-$ binding to provide a starting point for developing a geometric and electronic structural model for the putative Cu$^{II}_{M}$−OOH intermediate in the H-atom abstraction reaction of PHM. Possible electronic structure contributions of the Cu$^{II}_{M}$−OOH intermediate to reactivity are considered by correlation to the well-studied L$_3$Cu$^{II}$−OOH model complex (L$_3$ = [HB$(3\_Bu-5\_i$Prpz)$_3$]). The Met-S ligand of the Cu$_M$ site is found to contribute to the stabilization of the Cu$^{II}_{M}$−oxyl species, which would be a product of Cu$^{II}_{M}$−OOH H-atom abstraction reaction. This Met-S contribution could have a significant effect on the energetics of a H-atom abstraction reaction by the Cu$^{II}_{M}$−OOH intermediate.

Copper proteins play important roles in oxygen binding, activation, and reduction in biological systems (1). The copper active sites in these proteins catalyze enzymatic reactions that are often not possible in small molecule chemistry under ambient conditions. A number of these enzymes have binuclear copper active sites, which can be classified as either coupled or noncoupled binuclear sites according to the magnetic interactions between the two copper atoms.

The coupled binuclear copper proteins include hemocyanin, tyrosinase, and catechol oxidase (2). The binuclear copper sites in these proteins are strongly antiferromagnetically coupled through a side-on bridging ($\mu$-$\eta^2$:-$\eta^2$) peroxide ligand, which provides a direct mechanism for the initial two-electron reduction of dioxygen to peroxide (3). The interaction between the bridging peroxide and the two copper centers leads to their unique spectroscopic features (an intense peroxide $\pi^*$ to Cu charge transfer (CT) transition at ~350 nm and the lack of an EPR signal due to antiferromagnetic coupling) and activates the peroxide for electrophilic hydroxylation of enzymatic substrates (2, 4).

The noncoupled binuclear copper proteins include dopamine β-monoxygenase (DβM) and peptidylglycine α-hydroxylating monooxygenase (PHM) (5–8), which have two Cu sites separated by 11 Å with no bridging ligand and no magnetic interaction. The latter leads to a normal Cu$^{II}$ EPR signal with no spin–spin dipolar coupling. Both enzymes catalyze regiospecific and stereospecific C−H bond hydroxylation reactions of substrates incorporating one oxygen

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5 Abbreviations: PHM, peptidylglycine α-hydroxylating monooxygenase; PHMcc, catalytic core of PHM; DβM, dopamine β-monoxygenase; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; CT, charge transfer; ET, electron transfer; AcYVG, acetyl-Tyr-Val-Gly; L$_3$, [HB$(3\_Bu-5\_i$Prpz)$_3$]; LF, ligand field; XAS, X-ray absorption spectroscopy; EXAFS, extended X-ray absorption fine structure; ED, electric dipole; DFT, density functional theory; LUMO, lowest unoccupied molecular orbital.
atom from dioxygen and using ascorbate as the additional physiological reductant (eq 1).

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\begin{align*}
\text{(a)} & \\
\text{(b)} & 
\end{align*}
\]

The catalytic functions and mechanisms of DβM and PHM are very similar. The catalytic hydroxylation reaction occurs at one Cu site through a H-atom abstraction radical mechanism (9–12), and the other Cu site supplies an additional electron by long range (~11 Å) electron transfer (ET). Since the two Cu centers are not coupled, the mechanism of transporting the electron from one site to the other is unclear. A substrate-facilitated ET pathway (either through the substrate (13) or through protein residues brought closer upon substrate binding (14)) and a superoxide channeling mechanism (15) have been proposed for the intercopper ET. At the catalytic site, an as-yet unobserved CuII–OOH species has been widely proposed as the reactive intermediate for substrate hydroxylation (5). A peroxide species, CuII–O2– (or the alternative isoelectronic superoxide form CuI–O2–), was also proposed as a possibility (6). In the past, we have studied a mononuclear hydroperoxide model complex, L3CuII–OOH (L3 = [HB{3-fluoro-5-iPpz}]), hydrotitris(3-tert-butyl-5-isopropylpyrazolyl)borate, to gain insight into the possible reactivity of the proposed protein CuII–OOH intermediate (16). This L3CuII–OOH complex is not reactive for H-atom abstraction; spectroscopic and density functional calculations estimate this reaction to be endothermic by ~45 kcal/mol due to the high energy CuII–oxyl/CuIII–oxo product that would be generated after the homolytic O–O bond cleavage.

The crystal structure on the catalytic core of PHM (PHMcc) shows two inequivalent Cu sites in the protein separated by ~11 Å with no bridging ligation (13, 17). The CuM site has a distorted tetrahedral geometry with one methionine, two histidines, and a water-derived ligand. The CuH site has a nearly square planar geometry with three histidines and one water ligand. The substrate binds at a location close to the CuM site with the backbone hydrogen, which is abstracted in the enzymatic reaction, oriented toward at the CuM site, identifying the CuM as the catalytic site. The CuH is then associated with the ET site. Although their structures are clearly different, past spectroscopic studies (e.g., EPR) on oxidized PHM (and DβM) did not find any difference between the two Cu sites (18, 19). Controversy also exists concerning the structure of the oxidized CuM site where the spectroscopic results suggest a five-coordinate square pyramidal geometry in contrast to the four-coordinate tetrahedral geometry from crystallography (20). These would have very different ligand fields. However, Cu d–d transitions have not been observed for this enzyme.

In this study, we combine spectroscopic techniques and density functional calculations to define the geometric and electronic structures of the CuM and CuH site in resting oxidized PHM. In particular, low-temperature MCD allows the ligand field transitions to be observed for both Cu sites. Their geometric differences are correlated to their spectroscopic similarities through ligand field analyses to understand their indistinguishable spectra. A small molecule binding perturbation approach is found to differentiate the two CuII site contributions to the EPR and ligand field spectra allowing a correlation to their geometric differences. Parallel spectroscopic studies of structurally defined small model complexes are also presented to facilitate understanding the protein data. The highest occupied π* molecular orbitals of azide are analogous to the highest occupied π* orbitals of peroxy/hydroperoxide, both of which have pπ orbitals oriented orthogonal to the molecule axis (21, 22). Azide binding is thus used as an electronic and spectroscopic analogue of hydroperoxide to perturb resting PHM and provide a geometric and electronic basis for the putative CuII–OOH intermediate. The electronic structure description developed for the CuIII–OOH intermediate is correlated to the L3CuII–OOH model complex to obtain initial insight into its possible reactivity for H-atom abstraction and serves as a starting point for evaluation of the reaction energetics and mechanism of the enzymatic substrate hydroxylation. Importantly, these studies elucidate the nature of the activation of O2 for hydroxylation by a single Cu center.

1. EXPERIMENTAL METHODS

Materials. All reagents were of the highest grade commercially available and were used without further purification. The catalytic core of PHM (PHMcc) was expressed and purified as reported (14). Enzymatic activities were characterized using acetyl-Tyr-Val-Gly (AcYVG) as the substrate (14, 23). AcYVG was obtained from Stanford Protein and Nucleic Acid Biotechnology Facility. All spectroscopic measurements were done at pH ≈ 6.0 in deuterated MES/NaOH buffers. Protein samples were reconstituted with CuII by incubating with a slight excess of Cu(NO3)2 in 5–10 μM solution in the same deuterated MES buffer and then concentrated down to the desired protein concentration (~0.1 mM) in a Millipore Ultrafilter centrifugal filter. AcYVG, NaN2, and NaNO2 solutions were prepared in the same buffer and added into the protein solution for initial incubation and concentration for small molecule perturbation studies. All protein manipulations were done at ~4°C. Fifty to sixty percent glycerol-d1 was added as a glancing agent for low-temperature optical spectroscopy. From CD spectra, glycerol was found not to perturb the CuII site. Crystals of model complexes tetrakis(1,2-dimethylimidazole)CuII di(tetrafluoroborate) ([CuII(1,2-dimIm)4](BF4)2) and tetrakisimidazoleCuII sulfate ([CuII(Im)4](SO4)) prepared as in ref 24 were kindly provided by Prof. Harvey J. Schugar (Rutgers).

Spectroscopic Studies. EPR spectra were recorded on a Bruker EMX spectrometer in quartz tubes immersed in a liquid nitrogen finger Dewar. EPR parameters were obtained from simulations using XSope (25). Absorption spectroscopy was performed on a double beam spectrophotometer (Cary 500) equipped with a liquid helium cryostat (Janis Research Super Vari-Temp) or on a HP 8452A diode array spectrophotometer. Circular dichroism/magnetic circular dichroism (CD/MCD) data were collected on CD spectropolarimeters (JASCO J810 with a S20/S1 PM tube for the UV/
vis region and J200 with an InSb detector for the near-IR region) with sample compartments modified to accommodate magnetocryostats (Oxford Instruments, SM4000-7/8T). Due to the weak intensity of the PHM protein MCD signals, a magnetic field dependent but temperature independent background signal was often present. High-temperature 7 T spectra were thus subtracted from low-temperature 7 T spectra to remove this background and obtain the temperature-dependent MCD C-term signals. All spectra presented were normalized to the 5 K 7 T intensity using the Brillouin function for an $S = \frac{1}{2}$ system (26). Extinction coefficients were calculated on the basis of the sample Cu concentrations. Mull samples of the model complexes were prepared by dispersing finely ground powder of the complexes in poly-(dimethylsiloxane) (Aldrich) and sandwiching it between two quartz disks.

**DFT Calculations.** Density functional calculations were performed on a PC cluster using Gaussian 98 (27). All calculations were performed using the B3LYP functional (28) at the spin-unrestricted level. Two types of basis sets were used as implemented in the Gaussian 98 package. Geometry optimizations and frequencies were calculated with the LanL2DZ basis set. Single point energies were obtained with the triple-$\zeta$ TZV basis set. Wave functions were visualized in Molden (29) and analyzed with AOMix (30).

**Ligand Field Calculations.** Ligand field calculations were performed using a FORTRAN program written by Cecelia Campochiaro and modified by Elizabeth G. Pavel using the approach developed by Companion–Komarny (31, 32).

2. RESULTS

2.1. Spectroscopic. Figure 1A presents the ligand field (LF) region CD/MCD spectra of resting PHMcc. Only two electronic transitions are observed up to 30 000 cm$^{-1}$ in each spectrum, at $\sim$12 200 and $\sim$16 700 cm$^{-1}$, and form a dominant pseudo-A-term (i.e., temperature-dependent, derivative-shaped) feature in the MCD spectrum. Resting PHMcc is colorless, and no spectral feature is observed in its UV/vis/NIR absorption spectrum (data not shown). The X-band EPR spectrum of the resting PHMcc shows a typical tetragonal type 2 CuII signal with $g_z$ > $g_x, g_y$ > 2.0 indicating an $\chi^2$-$\gamma^2$ ground state (Figure 1B). Only one type of copper hyperfine coupling is resolved in the $g_z$ region, although there are two inequivalent Cu sites (CuM and CuH) in the protein. The $g_z$ and $A_z$ values determined from simulations are 2.288 and 175 $\times$ 10$^{-4}$ cm$^{-1}$, respectively (Table 1), consistent with those reported in the literature (33). In the presence of substrate, AcYVG, both transitions in the MCD spectrum did not shift in energy relative to those in the resting PHMcc (Figure 1A), indicating that the substrate binding does not perturb the geometries of the Cu sites in resting PHMcc. (The substrate concentrations used here are in the millimolar range, which ensures the complete substrate binding because the $K_M$(AcYVG) for PHMcc is $\sim$6 $\mu$M (14). Note that the intensity difference between the MCD spectra with and without substrate is due to baseline subtraction effects from weak MCD signals and some uncertainty in the sample Cu concentration.)

$N_2^-$ binding shifts the two LF transitions of the resting PHMcc Cu sites only slightly to $\sim$12 400 and $\sim$16 400 cm$^{-1}$ in the CD/MCD spectra of PHMcc + $N_2^-$ (Figure 1C).

![Figure 1](https://example.com/figure1.png)

**Table 1:** EPR $g$ and $A$ ($10^{-4}$ cm$^{-1}$) Values* and Calculated Ground-State Cu Covalency, $\beta^2$

<table>
<thead>
<tr>
<th></th>
<th>$g_z$</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$A_z$</th>
<th>$A_x$</th>
<th>$A_y$</th>
<th>$\beta^2$</th>
</tr>
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<tbody>
<tr>
<td>resting</td>
<td>2.050</td>
<td>2.060</td>
<td>2.288</td>
<td>9</td>
<td>12</td>
<td>157</td>
<td>0.70</td>
</tr>
<tr>
<td>w/N$_2$</td>
<td>2.040</td>
<td>2.060</td>
<td>2.246</td>
<td>2</td>
<td>25</td>
<td>160</td>
<td>0.66</td>
</tr>
<tr>
<td>w/NO$_2^-$</td>
<td>2.060</td>
<td>2.060</td>
<td>2.265</td>
<td>10</td>
<td>9</td>
<td>160</td>
<td>0.69</td>
</tr>
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</table>

* Complete simulation parameters are given in Table S1. $\beta^2$ values were obtained using the equation $\beta^2 = \frac{(g_x - g_y)^2}{A_x - A_y} + \frac{(g_y - g_z)^2}{A_y - A_z}$, where $P(\text{CuII}) \approx 400 \times 10^{-4}$ cm$^{-1}$, $\chi(\text{CuII}) \approx 0.43$, and $g_z = (g_x + g_y)/2$. The signs of experimental $A_z$ ($A_x$) values are taken as negative (35, 36).

However, new transitions are observed to higher energy in the charge-transfer region. The MCD spectrum shows three new transitions at 21 000 (+), 24 500 (--), and 28 600 (-- cm$^{-1}$) (Figure 2B). Two new transitions are also observed in the CD spectrum in the CT region at $\sim$25 800 (--) and $\sim$29 900 (+) cm$^{-1}$ (Figure 2B). In this energy region of the absorption spectrum of PHMcc + $N_2^-$, moderately intense charge-transfer features are observed at $\sim$26 000 cm$^{-1}$ with $\epsilon \approx 3400$ M$^{-1}$cm$^{-1}$ (Figure 2A), giving the solution a pale yellowish color. The energy and intensity of this absorption
band are consistent with the results of previously reported $N_3^–$ binding studies on $D_{4h}$ (34). Simultaneous Gaussian fit of the absorption, CD, and MCD spectra of PHMcc + $N_3^–$ in the CT region gives three transitions at energies of 21 950, 25 550, and 29 600 cm$^{-1}$ with extinction coefficients of $\sim$1050, 2550, and 2100 M$^{-1}$ cm$^{-1}$, respectively (Figure 2A). Since binding a single $N_3^–$ to a single $Cu^{II}$ can produce only two CT transitions (from the $N_3^–$ $\pi_{nb}^*$ and $\pi_{nb}$ orbitals), the presence of three bands indicates that two $N_3^–$ bind to the $Cu^{II}$ sites.

The X-band EPR spectrum of the $N_3^–$-bound form of PHMcc also shows only one type of tetragonal $Cu^{II}$ signal (Figure 1D). However, a smaller $g_z$ is observed (2.246, Table 1), and the line widths in the $g_z$ region become much narrower relative to those of resting PHMcc. Combined with the fact that three $N_3^–$ CT transitions are observed in absorption/CD/MCD, this indicates that both $Cu^{II}$ centers are perturbed by $N_3^–$ binding with one $N_3^–$ to each $Cu^{II}$. A similar EPR $g$ value perturbation by $N_3^–$ binding was also observed for $D_{4h}$ (34). Similar to resting PHMcc, the addition of substrate AcYVG to PHMcc + $N_3^–$ did not change the MCD spectra (Figure 1C and 2B), indicating that substrate binding does not perturb the Cu I sites of $N_3^–$-bound PHMcc. This is consistent with previous infrared studies on CO binding to the reduced PHMcc, which showed that substrate binding does not perturb the vibrational frequency of CO bound at the CuHg site (15).

$NO_2^–$ can bind to biological $Cu$ active sites (e.g., in nitrite reductase) and also form $Cu^{II}$ complexes in either monodentate or bidentate ligating mode (37). Previous studies using $NO_2^–$ as a perturbing small molecule provided a wealth of information about geometric and electronic structures of $Cu^{II}$ sites (38). $NO_2^–$ is thus used here to provide an additional handle to probe the $Cu^{II}$ sites in PHM. Since the protein sample was concentrated through a membrane filter, any exogenous $Cu^{II}$ was washed out, and the observed spectra are from protein-bound $Cu^{II}$. Possible binding of $NO_2^–$ to PHM other than the Cu sites cannot be determined here because the spectroscopic techniques used here only detect signals associated with the paramagnetic $Cu^{II}$ centers. The addition of 300 mM ($\sim$1500-fold) $NO_2^–$ significantly perturbed the MCD and EPR spectra of resting PHMcc. (The addition of 30 mM ($\sim$150-fold) $NO_2^–$ gave partial conversion.) Six transitions are now observed in the MCD spectrum at $\sim$6900 (+), $\sim$8900 (−), $\sim$10 700 (+), $\sim$12 350 (−), $\sim$14 200 (+), and $\sim$17 000 (−) cm$^{-1}$ (Figure 1E), as compared to the two-band spectrum of the resting PHMcc (Figure 1A). Since one $Cu^{II}$ site can at most contribute four $d$-$d$ transitions, the presence of six LF bands in the MCD spectrum indicates that both $Cu_M$ and $Cu_H$ sites in PHMcc are contributing and spectroscopically differentiated. In parallel, the X-band EPR spectrum of the $NO_2^–$-perturbed PHMcc shows two sets of $Cu^{II}$ hyperfine couplings in the $g_z$ region with approximately equal intensity, consistent with two inequivalent Cu sites (Figure 1F). The $g_z$ values of these two EPR signals, 2.265 and 2.298, are different from the two-band spectrum of the resting PHMcc (Figure 1A). Since one $Cu^{II}$ site can at most contribute four $d$-$d$ transitions, the presence of six LF bands in the MCD spectrum indicates that both $Cu_M$ and $Cu_H$ sites in PHMcc are contributing and spectroscopically differentiated. In parallel, the X-band EPR spectrum of the $NO_2^–$-perturbed PHMcc shows two sets of $Cu^{II}$ hyperfine couplings in the $g_z$ region with approximately equal intensity, consistent with two inequivalent Cu sites (Figure 1F). The $g_z$ values of these two EPR signals, 2.265 and 2.298, are different from the value of the resting PHMcc, further indicating that both Cu sites in the protein are perturbed. This is the first time that the two Cu sites in oxidized $D_{4h}$ or PHM could be spectroscopically differentiated. This is also consistent with the presence of three $N_3^–$ to Cu CT bands in the MCD spectrum of PHMcc + $N_3^–$ indicating two inequivalent $Cu^{II}$ sites (see section 3.3.1).

To facilitate the analysis of the PHMcc protein spectra, parallel spectroscopic studies were performed on two structurally defined tetragonal $Cu^{II}$ imidazole complexes. Figure 3 presents the low-temperature mull absorption and MCD spectra of the $[Cu^{II}(1,2$-dmIm)$_4](BF_4)_2$ complex, which has an approximately square planar geometry with four equatorial imidazole ligands. One dominant transition is observed in the absorption spectrum at $\sim$19 000 cm$^{-1}$ with a low-energy shoulder around 16 000 cm$^{-1}$ (Figure 3A). The extinction coefficients for these two transitions are about 50–100 M$^{-1}$ cm$^{-1}$ (24). Four transitions are observed in the MCD spectrum at $\sim$15 500 (−), $\sim$17 000 (+), $\sim$19 500 (−), and $\sim$23 000 (+) cm$^{-1}$ (Figure 3B). The middle two transitions in the MCD spectrum correspond to the strong absorption band at $\sim$19 000 cm$^{-1}$ (Figure 3A) and form a dominant...
MCD pseudo-A-term, very similar to the pseudo-A-term feature observed in the resting PHMcc MCD spectrum (Figure 1A). Similar spectral features are also observed in the MCD spectrum of the model complex [Cu(Il)(Im)4][SO4], which has a similar geometric structure to [Cu(Il)(1,2-dmIm)4]- (BF4)2 but with shorter axial metal–anion distances. (Figure S3).

2.2. Geometry Optimized Structures. To quantitatively correlate with the above spectroscopic data, geometric structure information for the Cu sites in PHM is required. Although past studies with X-ray absorption spectroscopy (XAS) (20) and crystallographic characterizations (13, 17) have provided consistent descriptions on the geometric structure of the CuM site in oxidized PHM, the protonation state of the bound water-derived ligand is not clear. For the CuM site, there is some controversy in the literature. While the crystal structure shows that the resting oxidized CuM site has a four-coordinate tetrahedral geometry (17), spectroscopic data (mainly EPR, XAS, and EXAFS) suggest a five-coordinate square pyramidal geometry with Met314 being the axial ligand (18, 20, 40). Further, no structural information is available for the N3- (i.e., peroxide analogue)-bound form of the PHM Cu sites or the putative CuIII-OOH intermediate. In this section, we computationally evaluate possible CuM and CuIII structural models using DFT methods to provide additional structural information for correlation with our spectroscopic results, and evaluation of existing structural models (Analysis section).

2.2.1. The Resting Cu Sites. The PHM CuM site has three protein ligands, Met314, His242, and His244 (13, 17). Including the possibilities of one to two water-derived ligands, four structural models were considered and geometry-optimized: [CuIII(Met)(His)2(H2O)(OH)]+, [CuIII(Met)(His)2(H2O)]2+, [CuIII(Met)(His)2(H2O)(OH)]2+, and [CuIII(Met)(His)2(OH)]+. The crystal structure coordinates were used as the starting geometries (13, 17). The methionine and histidines were modeled as ethylmethylthioether and methylimidazoles. The α-carbon positions of the methionine/histidine ligands were fixed in space during optimization to mimic the constraining effects of the protein backbone. (In very few cases, one small imaginary frequency (approximately −10 cm⁻¹) is present in the frequency calculations on the optimized structures due to these constraints. The corresponding vibrational modes involve dominantly motions of the α-carbons.) Without fixing the α-carbon positions, geometry optimizations resulted in different structures indicating the importance of incorporating the protein backbone effect on Cu active site geometries. The energy-optimized structures of the [CuIII(Met)(His)2(H2O)]2+ and [CuIII(Met)(His)2(H2O)(OH)]2+ models show a distorted square pyramidal and a slightly D2d distorted square planar geometry, respectively (Figure S4, parts A and B), both of which have the methionine as one of the equatorial ligands. This is not consistent with previous EXAFS results (20, 40), and the fact that no methionine to CuIII CT transition is present in the absorption spectrum indicates that the methionine has to coordinate at an axial position. These two models were thus eliminated from further evaluation. The [CuIII(Met)(His)2(H2O)(OH)]+ model has a square pyramidal geometry in the optimized structure with a long axial Cu–methionine bond (~2.81 Å) and two histidines, H2O, and OH− as the four equatorial ligands (1a in Figure 4A). The average equatorial metal–ligand bond length is ~2.03 Å. The structure is consistent with the proposed CuIII structural model based on spectroscopic studies (20). The geometry-optimized [CuIII(Met)(His)2(OH)]+ model could be described as either a trigonally distorted tetrahedral structure with methionine at the axial position or a distorted tetragonal structure with an open equatorial position opposite to His242 (1b in Figure 4A). The Cu–methionine bond is ~2.62 Å, and the average equatorial metal–ligand bond length is about 1.95 Å. This structure is consistent with the geometric description from the PHMcc crystal structure (17).

Parallel geometry optimizations were also performed on the possible models for the resting oxidized CuIII site, which has three histidine ligands from the protein backbone. Four structural models were evaluated with one or two water-derived ligands: [CuII(His)2(H2O)]2+, [CuII(His)(OH)]+,
[Cu$_{4}^{II}$(His)$_{3}$(H$_{2}$O)$_{2}$]$_{2}^{2+}$, and [Cu$_{4}^{II}$(His)$_{3}$(H$_{2}$O)(OH)]$^{+}$. The [Cu$_{4}^{II}$(His)$_{3}$(H$_{2}$O)(OH)]$^{+}$ model is not a stable structure, and the H$_{2}$O ligand dissociates from the Cu center. This model can thus be eliminated. The optimized structures of the other three models are shown in Figure 4D. The optimized [Cu$_{4}^{II}$(His)$_{3}$(H$_{2}$O)]$^{2+}$ model (4a) has a square planar geometry with a slight $D_{4d}$ distortion. The [Cu$_{4}^{II}$(His)$_{2}$(OH)]$^{+}$ model (4b) has a $D_{4d}$ distorted tetrahedral structure. The [Cu$_{4}^{II}$(His)$_{2}$(H$_{2}$O)$_{2}$]$_{2}^{2+}$ model (4c) has a square pyramidal geometry with a long axial Cu–H$_{2}$O bond ($r_{Cu-O,ax}$ ≈ 2.33 Å). The average equatorial metal–ligand bond lengths are about 2.01, 2.01, and 2.04 Å for 4a, 4b, and 4c, respectively (Figure 4D). These three resting oxidized Cu$_{4}$ models are all consistent with the PHMcc crystal structures (17), although structures 4a and 4b agree better with the previous spectroscopically defined tetragonal geometric description (20) since structure 4c is distorted toward a tetrahedral geometry. The possible structural models for the resting oxidized Cu$_{4}$M and Cu$_{4}$ sites (1a, 1b, 4a, 4b, and 4c) will be further evaluated on the basis of our spectroscopic results in the Analysis section.

2.2.2. The N$_{3}^{-}$-Bound Forms and the Putative Cu$^{III}$-OOH Intermediate. Possible structural models of the N$_{3}^{-}$-bound forms of the PHM Cu sites were also calculated and geometry-optimized for correlation with our spectroscopic results. Only two structural models are stable for the N$_{3}^{-}$-bound Cu$_{4}$ site, [Cu$_{4}^{III}$(Met)(His)$_{3}$(H$_{2}$O)(N$_{3}$)]$^{+}$ (2 in Figure 4B) and [Cu$_{4}^{III}$(Met)(His)$_{2}$(N$_{3}$)]$^{+}$ (Figure S4C). The [Cu$_{4}^{III}$(Met)(His)$_{3}$(N$_{3}$)]$^{+}$ model structure is very tetrahedral and could be eliminated on the basis of the spectroscopic features of N$_{3}^{-}$-bound PHMcc (vide infra). The optimized structure of the [Cu$_{4}^{III}$(Met)(His)$_{3}$(H$_{2}$O)(N$_{3}$)]$^{+}$ model (2) has a square pyramidal geometry with the Met-S being the axial ligand ($r_{Cu-S}$ ≈ 2.86 Å), and the N$_{3}^{-}$ is bound equatorially in an end-on fashion (average equatorial $r_{metal-ligand}$ ≈ 2.04 Å, Figure 4B). (The model structure [Cu$_{4}^{III}$(Met)(His)$_{2}$(OH)-(N$_{3}$)], where the bound H$_{2}$O at the resting Cu$_{4}$ site is replaced by N$_{3}^{-}$, is not stable during geometry optimization and leads to dissociation of the methionine ligand.) The only stable structure optimized for the N$_{3}^{-}$-bound Cu$_{4}$ site is the [Cu$_{4}^{III}$(His)$_{3}$(N$_{3}$)]$^{+}$ model (5, Figure 4E). It has a $D_{4d}$ distorted square planar geometry where the N$_{3}^{-}$ ligand binds terminally (average $r_{metal-ligand}$ ≈ 2.03 Å). In both N$_{3}^{-}$-bound structures 2 and 5, the N–N bond lengths are inequivalent with the remote N–N bond being ~0.05 Å shorter than the proximal one (Figure 4B,E). This is consistent with previous studies of N$_{3}^{-}$ bound terminaly to Cu$^{III}$ centers, which showed that the N$^{2-}$N$^{+}$=N resonance structure of the N$_{3}^{-}$ molecule is stabilized relative to the N$^{2-}$=N$^{+}$=N$^{-}$ structure upon binding to metal centers (21).

The N$_{3}^{-}$ ligand is a reasonable spectroscopic and electronic structure analogue of hydroperoxize because both have low-lying occupied $\pi$ orbitals ($\pi^{*}$ in hydroperoxide, $\pi^{*b}$ in N$_{3}^{-}$) that dominate the donor bonds (21, 22). Therefore, the geometry of the N$_{3}^{-}$-bound Cu$_{4}$ model (2) was used as the starting structure for geometry optimization of the widely proposed Cu$^{III}$M–OOH intermediate by substituting the end-on bound N$_{3}^{-}$ with the HOO$^{-}$ ligand. The final optimized structure of the [Cu$_{4}^{III}$(Met)(His)$_{3}$(H$_{2}$O)(OOH)]$^{+}$ model (3) is shown in Figure 4C. It has a square pyramidal geometry with Met-S as the axial ligand ($r_{Cu-S}$ ≈ 2.90 Å) and the hydroperoxide bound equatorially in an end-on fashion and

Scheme 1: Schematic Ligand Field State Energy Diagram for the Tetrakisimidazole Cu$^{III}$ Complex and Absorption and MCD Selection Rules

- The x and y axes defined here are approximately along the metal–ligand bonds in the tetrakisimidazole Cu$^{III}$ complexes and are 45° rotated (around z) from the conventional axis definition in the $D_{4d}$ point group to make the d$_{x^{2}-y^{2}}$ orbital highest in energy among the five d orbitals.
- The orbital label in parentheses indicates the location of the d hole of the Cu$^{III}$ center. The thickness of the arrows indicates the relative transition intensity. The absorption polarizations and MCD signal signs are indicated below the transition arrows.

hydrogen-bonded to the coordinated H$_{2}$O (average equatorial $r_{metal-ligand}$ ≈ 2.01 Å).

3. ANALYSIS

3.1. Ligand Field Analysis. 3.1.1. MCD Selection Rules and Spectral Assignments. In this section, we focus on the crystallographically defined [Cu$^{III}$(1,2-dmIm)$_{4}$]$^{2+}$ model complex to obtain ligand field absorption/MCD selection rules and make spectral assignments for correlation with the protein data. The CuN$_{4}$ core of the [Cu$^{III}$(1,2-dmIm)$_{4}$]$^{2+}$ complex has a slight $D_{4d}$ distortion from idealized $D_{4h}$ symmetry (24). This removes the inversion center of the molecule increasing the intensity of ligand field, d–d transitions, which would otherwise be parity forbidden. The ligand field state energy diagram for a Cu$^{III}$ complex with $D_{4h}(D_{4d})$ symmetry is shown in Scheme 1 (left column). There are three ligand field excited states, $2B_{1}(x^{2}-y^{2})$, $2E(xz,yz)$ (doubly degenerate), and $2A_{1}(z^{2})$ ($H_{2}$). The ground state is $2B_{2}(x^{2}-y^{2})$. Two transitions are electric dipole (ED) allowed in absorption. The transition to the doubly degenerate $2E(xz,yz)$ excited state is polarized along the $x$ and $y$ directions. The transition to the $2A_{1}(z^{2})$ state is allowed in the $z$ direction. The $2B_{2}(x^{2}-y^{2})$ → $2E(xz,yz)$ transition should be more intense in absorption as it gains intensity through configuration interaction with higher energy equatorial ligand to Cu CT transitions, which are also $x,y$ polarized. The higher CT intensity derives from the large orbital overlap between the ligand donor and the Cu $x^{2}-y^{2}$ acceptor orbital (Scheme 1, left column). Spin–orbit coupling within the $2E(xz,yz)$ state splits the degenerate pair in energy (by $|\lambda| \approx 830 \text{ cm}^{-1}$ for Cu$^{III}$) into two states that transform as $\Gamma_{s}$ ($|m_{J}| = 1/2$) and $\Gamma_{t}$ ($|m_{J}| = 3/2$) in the $D_{4d}$ double group (Scheme 1, center.
The absorption band at 

...column). The transitions to the 

(BF$_4$)$_2$ (Figure S1B), which would decrease the 2

significant intensity in the MCD spectrum. These two transitions, which are dominant in MCD.

...electric dipole transition moments to have

...rule requires a transition to have two perpendicular nonzero

...electric dipole transitions with the sulfate ligand relative to [CuII (1,2-dmIm)$_4$]-

...with corresponding Cu II M and Cu II H site models can be used

...parameters for each DFT-optimized Cu M and

...length (Table 2). (For cases where two model reference

...sulfate included in the distance data for the Cu II

...length (Å) 2 transition is also not observed. This suggests

...CuII (OH)(H$_2$(Bu-5-Prpz))$_3$] complex (Table S4) (16, 50). From ref 46. Because of the large Cu–S bond length difference with the two reference Cu–S bond lengths, the parameters here are obtained by linear extrapolations between the two sets of model reference values.

...The experimental observed LF transitions in the absorption spectrum of the [CuII(1,2-dmIm)$_4$](BF$_4$)$_2$ complex at 15 000–20 000 cm$^{-1}$ are low in intensity with extinction coefficients around 50–100 M$^{-1}$ cm$^{-1}$ (Figure 3A and section 2.1). Their energies are typical for CuII complexes in a close to square planar geometry (45). The strongest absorption band at ~19 000 cm$^{-1}$ can be assigned as the 3B$_2$(x$^2$−y$^2$) → 2E(xz,yz) transition (Figure 3A, Scheme 1, left column). The two MCD transitions at ~17 000 (+) and ~19 500 (−) cm$^{-1}$, which form the dominant pseudo-A term in the MCD spectrum, can be assigned as the two spin–orbit split components, 2T$_6$(xz,yz) and 2T$_8$(xz,yz), of the 2E(xz,yz) state, respectively (Figure 3B, Scheme 1 right column). The weak negative MCD transition at ~15 500 cm$^{-1}$ can be assigned as the transition to the 2T$_8$(z$^2$) state. Its corresponding absorption transition (2B$_1$(xy)) appears as a shoulder at ~16 000 cm$^{-1}$ (Figure 3A). The higher energy positive MCD feature at ~23 000 cm$^{-1}$ can be assigned as the transition to the 2T$_6$(z$^2$) state; its corresponding absorption feature is not experimentally resolved (Figure 3A,B). Similar spectral assignments can be applied to the absorption/MCD spectra of the [CuII(Im)$_4$](SO$_4$)$_2$ complex (Figure S3). The positive 2T$_6$(z$^2$) transition is not observed in the MCD spectrum (Figure S3B). This is likely due to additional axial interactions with the sulfate ligand relative to [CuII(1,2-dmIm)$_4$](BF$_4$)$_2$ (Figure S1B), which would decrease the 2T$_6$(z$^2$) state transition energy and result in its overlap with the xz/yz transitions, which are dominant in MCD.

The dominant pseudo-A-term feature in the MCD spectrum of resting PHMcc can be assigned as the CuII ligand field x$^2$−y$^2$ → xz/yz, 2B$_1$ → 2E, transitions, on the basis of the overall spectral similarity to the model complexes (Figures 1A, 3B, and 3B). The negative MCD CuII xy transition is not resolved for resting PHMcc, which could be due to its low intensity (Figures 3B and S2B) and the lower signal/noise ratio of the protein spectrum. The positive MCD CuII x$^2$−y$^2$ → z$^2$ transition is also not observed. This suggests possible axial interactions at the protein CuII sites, which could lower the z$^2$ transition energy and result in its overlap with the strong xz/yz transitions, as in the case of the [CuII(Im)$_4$](SO$_4$)$_2$ model (Figure S3B). Importantly, only one set of CuII xz/yz transitions are observed in the resting PHMcc MCD spectrum, while there are two inequivalent protein CuII sites (Cu$_{M3}$ and Cu$_{O4}$). This suggests that these two CuII sites have very similar d orbital splittings from their ligand field geometries. This is evaluated below.

### 3.1.2. Ligand Field Calculations

The DFT-optimized structures of possible Cu$_{M3}$ and Cu$_{O4}$ site models can be used to calculate their LF energy levels using the approach developed by Companion and Komarynsky (32) and applied to bioinorganic CuII and FeII sites (32, 44, 46). The ligands are treated as point charges, which perturb the CuII d orbitals due to electrostatic interactions based on the geometric arrangement of the ligands around the metal. The interactions of each ligand with the metal are parametrized by radial integrals $\alpha_2$ and $\alpha_3$ (32), which are obtained experimentally through fitting LF data on model complexes with defined geometries. The $\alpha_2$ and $\alpha_3$ parameters obtained in this way for imidazole, H$_2$O, OH$^-$, and Met-S ligands are given in Table 2 with corresponding Cu–ligand bond lengths. Individual ligand parameters for each DFT-optimized Cu$_{M3}$ and Cu$_{O4}$ models were scaled from the reference model values using the approximation that $\alpha_2$ and $\alpha_3$ are proportional to $1/r^6$ and $1/r^5$, respectively, where $r$ is the metal–ligand bond length (Table 2). (For cases where two model reference
values are available, the value associated with the bond length closest to the DFT-calculated one is used.) The $\alpha_2$ and $\alpha_4$ values for the histidine (imidazole), H$_2$O, and OH$^-$ ligands should be reasonable because the differences between the bond lengths in the calculated Cu$_{M}$/Cu$_H$ structures and those in the reference model systems are very small. The Met-S LF parameter values should also be reasonable because the Cu–S bond length in structure 1a is very close to that of the reference model complex, while the $\alpha_2$ and $\alpha_4$ values for Met-S in structure 1b ($r_{\text{Cu–S}} = 2.64$ Å) are bounded by the values of two model complexes with Cu–S distances of 2.30 and 2.90 Å.

The d–d transition energies obtained from the LF calculations on the DFT-optimized Cu$_M$ and Cu$_H$ model structures are given in Table 3 and compared with the experimental data. The $g$ values were also calculated using the LF-derived eigenfunctions and the perturbative approach outlined by Ballhausen (46, 51) and further corrected using Stevens’ orbital reduction factors to account for ligand covalency.

For the structural models of the PHM Cu$_M$ site (Figure 4A), structure 1a gives better agreement with both $xz/yz$ transitions than structure 1b, which underestimates their energies (Table 3). The predicted $xz/yz$ energy splitting is quite large for structure 1b (~4200 cm$^{-1}$) relative to that of structure 1a (~1800 cm$^{-1}$) and experiment (~2900 cm$^{-1}$). The LF calculated $g$ values for structure 1b give a significant rhombic splitting ($\Delta g_x$ = 0.10), which is not consistent with the experimentally observed axial EPR pattern of PHM. The large LF $xz/yz$ splitting and rhombic $g$ values of structure 1b result from its ligand field, which is more appropriately described as a distorted tetragonal structure with an open equatorial site (Figure 4A, section 2.2.1). The vacant equatorial position leads to a large inequivalence in the $x$ and $y$ directions giving the large $xz/yz$ splitting, while the overall distorted structure results in a significant $z^2$ mixing into the $x^2−y^2$ ground state (~19% from the LF calculation), which is the dominant factor leading to the rhombic EPR pattern. (DFT-calculated ground-state wave function for structure 1b (see section 3.2 and Figure 5A) gives ~4% $z^2$ mixing in the ground state, which corresponds to a rhombic splitting ($\Delta g_{\perp}$) of ~0.1 (36).) Therefore, the LF analysis indicates that the most reasonable Cu$_M$ structural model is [Cu$_{M}$(His)$_2$(Met)(H$_2$O)(OH)]$^+$ (1a, Figure 4A), which is square pyramidal with the Met-S as the axial ligand. This is consistent with the previous Cu$_M$ structural model based on XAS data (20). The DFT-optimized structure 1a provides a detailed model for Cu$_M$ based on spectroscopy.

For the structural models of the PHM Cu$_H$ site (Figure 4D), LF calculations on structure 4c underestimate the energies of both $xz/yz$ LF transitions, particularly the higher-energy component, which is underestimated by ~4000 cm$^{-1}$ (Table 3). Structure 4c can thus be eliminated. The results on structure 4b give good agreement for the low-energy component of the $xz/yz$ transitions, while the higher-energy component is underestimated by ~2600 cm$^{-1}$ (Table 3), arguing against structure 4b for the Cu$_H$ site. The LF results from structure 4a are in best agreement with experiment (Table 3). Therefore, the [Cu$_{H}$(His)$_3$(H$_2$O)]$^{2+}$ structure (4a in Figure 4D) is a reasonable model for the PHM Cu$_H$ site, where the Cu$^{II}$ center has a $D_{2d}$ distorted square planar geometry. This is consistent with the previously described Cu$_H$ structure based on both spectroscopy and crystallography (17, 20).

The LF calculated d–d transitions of structures 1a (Cu$_M$) and 4a (Cu$_H$) are plotted on the resting PHMcc MCD spectrum in Figure 5. The similarity between the d–d transition energies of the Cu$_M$ and Cu$_H$ sites is clearly demonstrated, especially for the two intense $xz/yz$ transitions. This is consistent with the spectroscopic data that show only one set of $xz/yz$ transitions forming the dominant pseudo-A term in the MCD spectrum of resting PHMcc. This appears to be inconsistent with the fact that there are two inequivalent Cu sites in the protein (Figure 1A), and the PHMcc + NO$^-$ data show that both sites contribute to the MCD spectrum (Figure 1E). The $z^2$ transitions for both Cu sites are located in the region of the strong negative MCD feature of the pseudo-A term and are thus not resolved experimentally. Further correlations between the Cu$_M$ 1a and Cu$_H$ 4a ligand field will be discussed in section 4.
are calculated to be very similar, about ~64% Cu character, more covalent than the experimental value determined from EPR (~70% Cu, Table 1). This agrees with past studies that the B3LYP functional overestimates ground-state covalencies of Cu complex (57). The important point here is that the ground-state Cu covalencies of the CuM 1a and CuH 4a sites are essentially equal. Together with their similar LF transition energies, both the CuM and CuH sites should exhibit very similar EPR g and A values, since the g values are only dependent on LF energies and covalencies and the A values depend on the g values and covalency of the ground state. This is indeed reflected experimentally in the EPR spectrum of resting PHMcc where only one CuII EPR signal is observed (Figure 1B).

3.3. Electronic Structure Description of the Putative CuII–OOH Intermediate. 3.3.1. N3–-Bound PHM. N3– was used as a spectroscopic and electronic analogue of OOH– to probe the Cu sites in resting PHMcc. The EPR spectrum of PHMcc + N3– shows one type of tetragonal Cu EPR signal, similar to that of resting PHMcc but with a smaller gz value, indicating that both Cu sites in the protein are perturbed (Figure 1D, Table 1, section 2.1). Two intense (and one weak) CT transitions are observed in the absorption spectrum of PHMcc + N3– at 25 550 cm−1 (ε ≈ 2550 M−1 cm−1) and 29 600 cm−1 (ε ≈ 2100 M−1 cm−1) with two corresponding negative bands in the MCD spectrum (Figure 2). The absorption intensities of these two CT transitions require significant overlap between N3–πab and Cu x2–y2 orbitals (27). N3– thus must bind equatorially to the Cu sites, one to each Cu site, since one N3– molecule can only contribute to one intense CT absorption transition. The DFT-optimized structures CuIII–N3– 2 (Figure 4B) and CuII–N3– 5 (Figure 4E) are consistent with the spectroscopic results; both have one equatorial N3– bound to the Cu centers. Specifically, the higher energy transition at 29 600 cm−1 can be associated with the N3–πab → CuII– x2–y2 transition, while the lower energy transition can be assigned to the N3–πab → CuII– x2–y2 transition. The energy difference comes from their difference in Cu coordination numbers (CuIII–N3– 2, five-coordinate; CuII–N3– 5, four-coordinate; Figure 4B,E) where the higher coordination number leads to the higher energy CT transition due to the shift in the Cu d manifold to higher energy from more ligand repulsion and a lower Cu effective nuclear charge (Zeff). In correlation to the NO2− binding studies (Section 2.1), the inequivalent energies of the two N3–πab → Cu CT bands observed experimentally provide further support for the inequivalence of the CuM and CuH sites. The CT band at 21 950 cm−1 with low intensity (ε ≈ 1050 M−1 cm−1) can be associated with the N3–πy → Cu transition of one Cu site. The N3–πy orbital is perpendicular to the Cu x2–y2 orbital with little orbital overlap and thus low in intensity.

The Cu LF x2/y2 transitions in the MCD spectra are only slightly perturbed by N3– binding to PHMcc (Figure 1A,C), indicating that the Cu site geometries in the N3–-bound form remain tetragonal and similar to resting PHMcc. This is consistent with the DFT-optimized structures CuIII–N3– 2 and CuII–N3– 5 (Figure 4B,E), which have square pyramidal and D2d distorted square planar geometries, respectively, paralleling the resting CuM 1a and CuH 4a structures (Figure 4A,D). The LF data of PHMcc + N3– also rule out
the other computationally stable structure ([Cu$^{III}$](Met)(His)$_2$-(N$_3$)]$^+$, Figure S4C) because it has a close to tetrahedral geometry.

3.3.2. Electronic Structure of the Putative Cu$^{III}$–OOH Intermediate. The DFT-optimized structure 3 for the putative Cu$^{III}$–OOH intermediate has a square pyramidal geometry (Figure 4C). This is very similar to the spectroscopically defined Cu$^{III}$–N$_3^-$ structure 2, which was used as the starting geometry with substitution of the N$_3^-$ by OOOH, reflecting the electronic analogy between N$_3^-$ and OOOH. The OOOH binds in an end-on fashion to the Cu$^{III}$ center. (The deprotonated water ligand form of Cu$^{III}$–OOH, [Cu$^{III}$(Met)(His)$_2$(OH)(OOH)], was also geometry-optimized and found to be ~25 kcal/mol higher in free energy.) The ground-state wave function of this Cu$^{III}$–OOH 3 intermediate is shown in Figure 6C, left, and consists mainly of the Cu $x^2$–$y^2$ orbital, similar to that of resting CuM 1a (labeled $(x^2–y^2)–\pi^*_{xy}$, Figure 6A). The OOOH $\pi^*$ orbital is in the Cu–O–O plane and forms a dominant but not very coherent pseudo-ơ interaction with the Cu$^{III}$ $x^2$–$y^2$ orbital, since there is only a total of 19% OOOH character in the wave function.

The OOOH $\pi^*$ component of the ground-state wave function is highly polarized toward the Cu (ligating oxygen, ~17%; remote oxygen, ~2%), similar to the behavior observed in Cu$^{III}$–OOH model complexes (16, 58). This polarization is mainly due to the effect of protonation and leads to a strengthened O–O bond (16, 58). The OOOH $\sigma^*$ orbital (Figure 6C, right) is high in energy (~3 eV higher than the spin-down LUMO) and also polarized toward the Cu (ligating oxygen, ~40%; remote oxygen, 13%), similar to that of Cu$^{III}$–OOH model complexes (16, 58). Possible electronic structure contributions of this putative Cu$^{III}$–OOH 3 intermediate to reactivity will be evaluated below.

4. DISCUSSION

A combination of spectroscopic characterizations and DFT calculations has defined the geometric and electronic structures of the CuM and CuH sites in resting PHM. The CuM site has a square pyramidal geometry with a long axial Cu–methionine bond and two histidines, H$_2$O, and OOOH as the equatorial ligands (structure 1a, Figure 4A). The CuH site has a slightly D$_2h$ distorted square planar geometry with three histidines and H$_2$O as the equatorial ligands (structure 4a, Figure 4D). These two sites have very similar ligand field transition energies and ground-state covalencies leading to their indistinguishable contributions in optical and EPR spectra (Figures 1A,B and 6A,B). These spectroscopic properties are the result of the metal–ligand interactions at these LF geometries. The spectral similarities of CuM and CuH appear to contradict the fact that these two sites have different number and type of ligands; relative to the CuH site, the CuM site has an additional axial Met-S ligand and an equatorial OH$^-$ in place of a histidine. We have thus performed a LF analysis for a series of intermediate structures correlating the CuM and CuH sites.

Figure 7 (left) presents the $d$ orbital splittings from the LF calculation on CuH 4a (section 3.1.2). The highest energy $d$ orbital is $x^2–y^2$ with the $xy$, $xz/yz$, and $z^2$ orbitals at decreasing energy, reflecting the close to square planar geometry of the CuH site. Figure 7 (IS.1) shows the $d$ orbital splittings of an intermediate structure derived from CuM (structure 1a in Figure 7, right), where the axial methionine ligand is removed, and the OH$^-$ ligand is replaced by a histidine to have the same ligand set as the CuM site. (The $\alpha_2$ and $\alpha_4$ parameters for the substituted histidine here were taken as the average values of the three histidine ligands of the CuM site.) The Cu atom is above the ligand plane in a square pyramidal geometry. This out of plane distortion from planar geometry mainly results in the lowering of the $x^2–y^2$ orbital and a slight increase of the $xz$ and $yz$ orbital energies, leading to the decreased energy splittings of the $x^2–y^2/xy$ and $x^2–y^2/(xz, yz)$ orbitals. Having an equatorial OH$^-$ ligand instead of a histidine in this intermediate structure mainly elevates the energy of the $x^2–y^2$ orbital due to the larger donor interaction of OH$^-$ relative to histidine ($\alpha_2$ and $\alpha_4$ in Table 2), resulting in increased energy splittings of the $x^2–y^2/xy$ and $x^2–y^2/(xz, yz)$ orbitals (Figure 7, IS.2). The presence of the axial Met-S ligand at the CuM site exerts an additional interaction in the $z$ direction and shifts the $z^2$, $xz$, and $yz$ orbitals up in energy, while again decreasing the energy splittings of the $x^2–y^2/xy$ and $x^2–y^2/(xz, yz)$ orbitals (Figure 7, right). Thus, the effects of the square pyramidal distortion and addition of the axial methionine ligand at the CuM site oppose the effects of the strong donating equatorial OH$^-$ ligand on the Cu $d$ orbital splitting pattern, leading to LF transition energies similar to those of the CuH site (Figure 7, left and right, and Figure 5). The only significant difference is the energy of the $z^2$ orbital, which is higher for the CuM site due to the axial Met-S interaction. However, the $z^2 \rightarrow x^2–y^2$ transition does not contribute to the EPR $g$ values (for a $x^2–y^2$ ground state, $g_z = 2.0 – 8\alpha_4/\beta_2^2\Delta E_{\text{xy}}$ and $g_{x,y} = 2.0 – 2\gamma_1/\beta_1^2\Delta E_{\text{xy}}$, where $\alpha_4$, $\beta_1$, and $\gamma_1$ are the $Cu \ xy$, $x^2–y^2$, and $xy$ orbital contributions in the LF wave functions and $\Delta E_{\text{xy}}$ and $\Delta E_{\text{yz}}$ are the $xy$ and $xz/yz$ to $x^2–y^2$ transition energies (59)) and is not observable in the
Azide binding studies on resting PHM have provided a geometric and electronic structure model for the azide-bound form, Cu\textsuperscript{II}M\textsubscript{3}−N\textsubscript{3}− (structure 2, Figure 4B), and facilitated development of a structural model for the putative Cu\textsuperscript{II}−OOH species (structure 3, Figure 4C) previously proposed as a reactive intermediate in catalysis by PHM and D/JM (5, 7, 60). This Cu\textsuperscript{II}M−OOH model provides an electronic structure description of the Cu−hydroperoxide and the O−O bonding interactions (section 3.3.2). The \( \pi_r^* \) orbital of the bound OH\textsubscript{2}−ligand forms a dominant pseudo-\( \sigma \) interaction with the Cu\textsuperscript{II} \( x^2−y^2 \) orbital; the resulting interaction is not very covalent (61% Cu + 19% OH\textsuperscript{−}, Figure 6C, left). This bonding description is different from the results on a previously characterized mononuclear L3Cu\textsuperscript{II}−OOH model complex ([L = [HB(3-tBu-5-iPrz)\textsubscript{2}]] (16), where the \( \pi_r^* \) orbital of the OH\textsuperscript{−} ligand undergoes a dominant \( \pi \) donor interaction with the Cu\textsuperscript{II} \( x^2−y^2 \) orbital, which results in a more covalent Cu−O bond (58% Cu + 31% OH\textsuperscript{−}, Figure 6D). The difference in the Cu−O bonding interactions between Cu\textsuperscript{II}−OOH and L3Cu\textsuperscript{II}−OOH derives from their different LF geometries. The Cu\textsuperscript{II}−OOH intermediate is five-coordinate and has a square pyramidal geometry resulting in a Cu \( x^2−y^2 \) orbital lobe oriented along the Cu−O bond and thus a pseudo-\( \sigma \) bonding interaction with the OH\textsuperscript{−} ligand (Figure 6C, left). The L3Cu\textsuperscript{II}−OOH model complex is four-coordinate and has a trigonally distorted tetrahedral geometry where the Cu\textsuperscript{II} \( x^2−y^2 \) orbital has two adjacent lobes bisected by the Cu−O bond leading to a \( \pi \) bonding interaction with the OH\textsuperscript{−} ligand (Figure 6D). The higher coordination number of the Cu\textsuperscript{II}−OOH species raises the energy of the Cu d manifold due to the increased ligand repulsion and lower \( Z_{\text{eff}} \), increasing the energy separation between the Cu d and the OH\textsuperscript{−} ligand valence orbitals. This results in the less covalent, weaker Cu−O bond in Cu\textsuperscript{III}−OOH relative to that of the L3Cu\textsuperscript{II}−OOH complex. In contrast to the Cu−O bond, the O−O bond strength in Cu\textsuperscript{II}−hydroperoxide species is mainly determined by the protonation, which increases the bond strength, and is thus not significantly affected by the Cu−O interaction (16, 58). The internal H-bond structure feature of the Cu\textsuperscript{III}−OOH intermediate 3 (Figure 4C) will contribute to the energetics of its formation (61), which is addressed in another study (62).

The bonding description of the putative Cu\textsuperscript{II}−OOH species provides initial insight into its possible reactivity in the H-atom abstraction reaction, which occurs in PHM and D/JM catalysis (5, 9−12). This would involve the homolytic cleavage of the O−O bond generating a Cu\textsuperscript{II}−oxy\textsubscript{1}/Cu\textsuperscript{III}−oxo product species. Our previous studies on the L3Cu\textsuperscript{II}−OOH model have shown that this reaction is endothermic by ~45 kcal/mol due to the high-energy product generated, which is best described as a Cu\textsuperscript{II}−oxy rather than a Cu\textsuperscript{III}−oxo species (16). The high Cu\textsuperscript{II}/Cu\textsuperscript{III} redox potential and the inability of the pyrazole ligand to stabilize the Cu\textsuperscript{II}−oxyl species are the main factors contributing to this large \( \Delta E \). Comparatively, the Cu\textsuperscript{II}−OOH species has a higher coordination number than the L3Cu\textsuperscript{II}−OOH complex, which would contribute to stabilizing the Cu\textsuperscript{II}−oxyl species through charge donation. However, its less covalent Cu−O interaction would tend to destabilize the product. The equatorial histidine ligands in Cu\textsuperscript{III}−OOH are very similar to the pyrazole ligands in L3Cu\textsuperscript{II}−OOH in electronic properties (63) and would not assist in product stabilization, nor would the bound water ligand, which is an even poorer donor ligand than histidine. (The possibility that the water ligand would deprotonate upon the homolytic O−O bond cleavage and the resulting OH\textsuperscript{−} ligand would possibly stabilize the Cu\textsuperscript{III}−oxyl intermediate is evaluated by DFT geometry optimizations. The methionine ligand dissociates for the Cu\textsuperscript{III}−oxyl intermediate with OH\textsuperscript{−} ligand, and the total energy goes up by > 9 kcal/mol relative to the Cu\textsuperscript{III}−oxyl intermediate with H\textsubscript{2}O ligand.) However, the Cu\textsuperscript{II}−OOH species has a Met-S ligand, which is a much better donor ligand and easier to oxidize than the pyrazoles of the L3Cu\textsuperscript{II}−OOH complex. Although the Met-S has virtually no contribution to the ground-state wave function of the Cu\textsuperscript{III}−OOH species (Figure 6C, left), it may contribute to the stabilization of the Cu\textsuperscript{III}−oxyl product upon Cu\textsuperscript{II}−OOH H-atom abstraction on substrate. The geometry-optimized structure of the Cu\textsuperscript{III}−oxyl species indicates that the Met-S ligand is in fact in an equatorial position and becomes partially oxidized relative to that in the Cu\textsuperscript{III}−OOH intermediate, contributing to the stabilization of the Cu\textsuperscript{III}−oxyl species (62). This Met-S contribution could have significant effects on the energetics of the H-atom abstraction reaction by the Cu\textsuperscript{III}−OOH intermediate. The energetics and reaction barrier of Cu\textsuperscript{II}−OOH H-atom abstraction are evaluated in another study (62), along with another alternative reaction pathway for the PHM reaction.

NOTE ADDED IN PROOF:

A Cu\textsuperscript{II}−superoxide species has recently been proposed to be the reactive CuO\textsubscript{2}− intermediate for H-atom abstraction in D/JM chemistry (64). The reaction thermodynamics and energy barriers for the H-atom abstraction reaction of Cu\textsuperscript{III}−superoxo and Cu\textsuperscript{II}−OOH species in PHM have been evaluated in ref 62.

SUPPORTING INFORMATION AVAILABLE

Structures of [Cu\textsuperscript{II}(1,2-dmIm)\textsubscript{2}(ClO\textsubscript{4})\textsubscript{2}] and [Cu\textsuperscript{II}(Im)\textsubscript{4}](SO\textsubscript{4}) complexes, Gaussian-resolved CD/MCD spectra of PHMcc + N\textsubscript{3}−, abs/MCD spectra of [Cu\textsuperscript{II}(Im)\textsubscript{4}](SO\textsubscript{4}), additional geometry-optimized structures, ground-state wave functions of Cu\textsubscript{III} 1b and Cu\textsubscript{II} 4b, complete EPR simulation parameters, ligand field fit parameters to [Cu\textsuperscript{II}(Im)\textsubscript{4}]\textsuperscript{2+}, [Cu\textsuperscript{II}(H\textsubscript{2}O)\textsubscript{6}]\textsuperscript{2+}, and [Cu\textsuperscript{II}(OH)(HB(3-tBu-5-iPrz)\textsubscript{2})] models, and coordinates of DFT-optimized structures. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


