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A macrocyclic quinol-containing ligand enables high catalase activity even with a redox-inactive metal at the expense of the ability to mimic superoxide dismutase†

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Previously, we found that linear quinol-containing ligands could allow manganese complexes to act as functional mimics of superoxide dismutase (SOD). The redox activity of the quinol enables even Zn(II) complexes with these ligands to catalyze superoxide degradation. As we were investigating the abilities of manganese and iron complexes with 1,8-bis(2,5-dihydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane (H₄qp4) to act as redox-responsive contrast agents for magnetic resonance imaging (MRI), we found evidence that they could also catalyze the dismutation of H₂O₂. Here, we investigate the antioxidant behavior of Mn(II), Fe(II), and Zn(II) complexes with H₄qp4. Although the H₄qp4 complexes are relatively poor mimetics of SOD, with only the manganese complex displaying above-baseline catalysis, all three display extremely potent catalase activity. The ability of the Zn(II) complex to catalyze the degradation of H₂O₂ demonstrates that the use of a redox-active ligand can enable redox-inactive metals to catalyze the decomposition of reactive oxygen species (ROS) besides superoxide. The results also demonstrate that the ligand framework can tune antioxidant activity towards specific ROS.

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Introduction

High concentrations of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and superoxide (O₂^{•−}) are capable of damaging biomolecules, and the accumulation of these species has been linked to a wide array of health conditions.^{1–5} In response, the body produces a variety of antioxidants to manage ROS concentrations. These include superoxide dismutases (SODs), which catalyze the conversion of O₂^{•−} to O₂ and H₂O₂, and catalases, which promote the dismutation of H₂O₂ to O₂ and H₂O. The high activities of these enzymes have motivated us and other researchers to develop small molecules capable of replicating this catalysis. Such compounds could potentially be used to bolster the body's defenses against ROS and treat health conditions associated with oxidative stress.

In previous work from our laboratories, we found that linear polydentate ligands with 1,4-hydroquinone (quinol) groups could be used as the organic components in a variety of SOD mimics (Scheme 1).^{6–10} A manganese complex with H₂qp1 displayed activity that was comparable to those of the most effective SOD mimetics reported to date: manganese complexes with pentaazamacrocyclic and cationic porphyrin ligands.^{11–14} The redox activity of the quinol group enables it to act as the redox partner for O₂^{•−} and can allow SOD mimicry even without a redox-active metal. Although the ligands by themselves are inactive, the Zn(II) complexes with H₂qp1 and H₄qp2 are functional SOD mimics, with the latter ligand resulting in higher activity.^{9,10}



Scheme 1 Linear polydentate quinol-containing ligands from prior work.

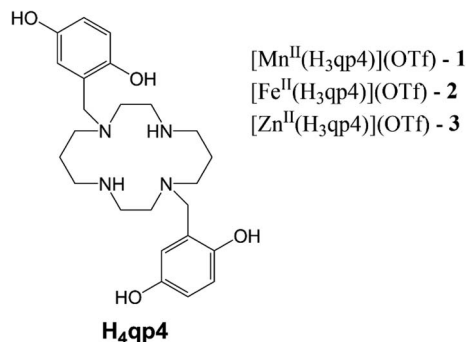
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Scheme 2 Structure of H₄qp4 ligand and formulations of the discussed coordination complexes.

More recent work has focused on the macrocyclic ligand 1,8-bis(2,5-dihydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane (H₄qp4, Scheme 2).^{15,16} Our initial interest in this molecule was as a component in highly water-stable magnetic resonance imaging (MRI) contrast agent sensors for H₂O₂. When the quinols are oxidized to *para*-quinones, water molecules displace these groups, increasing the T₁-weighted relaxivity (r₁) of its Mn(II) complex, [Mn^{II}(H₃qp4)](OTf) (1).¹⁵ Although quinol oxidation and an accompanying enhancement in r₁ also occur when the Fe(II) complex, [Fe^{II}(H₃qp4)](OTf) (2), reacts with H₂O₂, it is instead metal oxidation that is primarily responsible for the increase in relaxivity.¹⁶ For both 1 and 2, we found that the oxidation of the quinols and the accompanying activation of the sensor occur more slowly in higher concentrations of H₂O₂.^{15,16} This led us to speculate that the initial reactions with H₂O₂ may be generating a higher-valent metal species that can either react intramolecularly to oxidize the ligand or intermolecularly with a second equiv. of H₂O₂ (Scheme 3).¹⁵ The intermolecular activity depletes H₂O₂, thereby mimicking catalase. The intramolecular reaction was proposed to be inherently slower, only proceeding to a noticeable extent after the [H₂O₂] decreases enough to make the intermolecular reaction less competitive.

Replicating the activity of catalase enzymes has proven to be challenging, and the best small molecule mimics reported thus far have displayed modest reactivity with H₂O₂. Two metrics are commonly used to assess catalysis: the rate constant for the reaction between H₂O₂ and the antioxidant and the turnover number (TON).^{17–27} Comparisons are often difficult to make since the reaction conditions and protocols can vary considerably. The highest rate constant for H₂O₂ degradation corresponded to a second-order reaction with an Fe(III) complex with a fluorinated corrole (k₂ = 4300 M⁻¹ s⁻¹ in 37 °C pH 7.4 phosphate buffer).²⁷ The highest TON corresponded to a manganese porphyrin complex (TON = 12.54 in 25 °C pH 7.8 Tris buffer).²²

In the present manuscript, we thoroughly investigate the abilities of the Mn(II) and Fe(II) complexes with H₄qp4 to mimic both SOD and catalase. We also prepare and evaluate the antioxidant properties of a Zn(II) complex with H₄qp4: [Zn^{II}(H₃qp4)](OTf) (3). We find that although the SOD activities are severely attenuated relative to those of complexes with linear quinol-containing ligands, all three H₄qp4 complexes act as highly potent catalase mimics, with activities that greatly exceed those exhibited by most other reported small molecule mimics of these enzymes.^{17–27} The Zn(II) complex is further notable in that it represents the first instance, to the best of our knowledge, where a coordination complex with a redox-inactive metal ion successfully catalyzed the degradation of H₂O₂.

Experimental section

Materials

All chemicals and solvents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. All deuterated solvents were bought from Cambridge Isotopes. Diethyl ether (ether) and methanol (MeOH) were bought from Fisher. Methylene chloride (CH₂Cl₂) was purchased from Mallinckrodt Baker. 1,8-Bis(2,5-dihydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane (H₄qp4), [Mn^{II}(H₃qp4)](OTf), and [Fe^{II}(H₃qp4)](OTf) were synthesized and purified as previously described.^{15,16}



Scheme 3 Previously proposed mechanism for competing H₂O₂ degradation (blue) and quinol oxidation (red). The above graphic was slightly modified from one that originally appeared in ref. 15.



Instrumentation

All nuclear magnetic resonance (NMR) data were collected on a 500 MHz AV Bruker NMR spectrometer. All NMR resonance peak frequencies were referenced to internal standards. UV/vis were collected on a Varian Cary 50 spectrophotometer and analyzed using software from the WinUV Analysis Suite. Electron paramagnetic resonance (EPR) data were obtained on a Bruker EMX-6/1 X-band EPR spectrometer operated in the perpendicular mode. The EPR data were subsequently analyzed and processed with the program EasySpin. All EPR samples were run as frozen solutions in quartz tubes. High-resolution mass spectrometry (HR-MS) data were obtained at the Mass Spectrometer Center at Auburn University on a Bruker Microflex LT MALDI-TOF mass spectrometer *via* direct probe analysis operated in the positive ion mode. Infrared spectroscopy (IR) data were obtained with a Shimadzu IR Prestige-21 FT-IR spectrophotometer. Cyclic voltammetry (CV) was performed under N₂ at 294 K using an Epsilon electrochemistry workstation (Bioanalytical System, Inc.), a gold working electrode, a platinum wire auxiliary electrode, and a silver/silver(I) chloride reference electrode. All elemental analyses (C, H, N) were performed by Atlantic Microlabs (Norcross, GA); samples were dried under vacuum and placed under a N₂ atmosphere prior to shipment.

X-ray crystallography

Crystallographic data for [Zn^{II}(H₃qp4)](OTf) (3) were collected using a Bruker D8 VENTURE κ-geometry diffractometer system equipped with a Incoatec IμS 3.0 microfocus sealed tube and a multilayer mirror monochromator (Mo Kα, λ = 0.71073 Å). Diffraction data were integrated with the Bruker SAINT software package using a narrow-frame algorithm. Data were corrected for absorption effects using the Multi-Scan method (SADABS). The structure was solved and refined using the Bruker SHELXTL Software Package. Selected crystallographic data are presented in the ESI and can be found in the Cambridge Structural Database (number 2173563†).

Antioxidant assays

The ability of coordination complexes to catalyze the degradation of O₂^{•−} was initially screened using the hypoxanthine/xanthine oxidase/lucigenin assay.^{28,29} The reaction between hypoxanthine and xanthine oxidase generates O₂^{•−}, which can then subsequently react either with lucigenin to provide a luminescent response or an antioxidant. The extent to which various concentrations of an antioxidant eliminate the lucigenin response provides a quantitative measure of its activity. These assays were performed with 1 mL of saline solutions buffered with 50 mM phosphate to pH 7.2. The solutions also contained 50 μM hypoxanthine, 0.005 U mL^{−1} xanthine oxidase (Calbiochem), 5 μM dark adapted lucigenin, and the tested antioxidant in concentrations ranging from 0.1 nM to 10 μM. Reactions were carried out at room temperature (RT) and were initiated by the addition of xanthine oxidase to the hypoxanthine-containing solution. The copper/zinc superoxide

dismutase isolated from bovine erythrocytes (0.0001–10 U mL^{−1}, Calbiochem) was used as a positive control (IC₅₀ = 40 nM).³⁰ Luminescence was measured using a TD-20/20 (Turner Designs) luminometer and expressed as relative light units (RLU). Luminescence was measured for four 10 s integrations after an initial delay of 3 s. The four RLU values were averaged, and each concentration was expressed as a percent of that produced in the presence of the vehicle. Each measurement within an individual run was performed in triplicate, and each assay was repeated three times.

Kinetic assessment of superoxide dismutase activity

The ability of the H₄qp4 complexes to catalytically degrade superoxide was tested by a direct method using stopped-flow techniques described in a previous publication from one of our laboratories.³¹ Experiments were carried out using syringes 1, 2, and 3 on a Biologic SFM-400 instrument that was equipped with an Energetiq LDLS ENQ EQ-99-FC laser driven light source and a J&M TIDAS diode array detector (integration time = 0.5 ms, λ = 180–724 nm). The source of superoxide was commercially available KO₂ dissolved in dry and non-buffered DMSO ([O₂^{•−}] ≈ 1–2 mM). Complexes 1–3 were each tested at four different concentrations between 0.9 and 9 μM in aqueous solutions buffered with HEPES or sodium phosphate to either pH 7.4 or pH 8.1. The ionic strength of all solutions was 111 mM. The aqueous solution containing the studied coordination complex was mixed in a 9 : 1 ratio with the superoxide solution in DMSO using a high-density mixer. The initial concentration of the superoxide is determined from the intensity of the UV band at 250 nm as assessed immediately after stopped-flow mixing of the superoxide DMSO solution with the appropriate buffer. The 250 nm band is characteristic for superoxide and has well-known and -established molar extinction coefficients for each buffer and pH used.³² In each experiment, the concentration of superoxide exceeded that of the metal-containing catalyst by at least ten-fold to ensure catalytic conditions. Millipore water was used for the preparation of the buffer solutions. All of the prepared buffers were treated with Chelex 100 sodium exchange resin for at least 12 h before use in order to remove adventitious metal ions. The data analysis was performed using the BioKine V4.66 software. Each reported *k*_{obs} value is the average of at least 10 measurements. The reported *k*_{cat} values were determined from the slope of the *k*_{obs} vs. [SODm] plot. The presence of H₂O₂ was qualitatively confirmed using Baker Testrips for Peroxides.

Kinetic assessment of catalase and peroxidase activity

The abilities of the antioxidants to catalyze H₂O₂ degradation were initially evaluated by monitoring the decrease in the absorbance of H₂O₂ at 240 nm ($\epsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$)³³ over time. These measurements were performed in 200 mM phosphate solutions buffered to pH 7.0 that contained 100 nM of the tested compound and 1–500 mM H₂O₂ at RT. The changes in the UV/vis data were followed using a Shimadzu UV-1601 spectrophotometer (Columbia, MD). Under the described conditions, we observed a single hyperbolic phase and could fit



the data to a standard Michaelis–Menten equation to obtain apparent k_{cat} and k_{on} values (Eq. (1)).

$$\frac{v_o}{[I]_T} = \frac{k_{\text{cat}}[\text{H}_2\text{O}_2]}{k_{\text{cat}}/k_{\text{on}} + [\text{H}_2\text{O}_2]} = \frac{k_{\text{cat}}[\text{H}_2\text{O}_2]}{K_M + [\text{H}_2\text{O}_2]} \quad (1)$$

The catalase activity was more stringently quantitated by polarographically following O_2 production using a Clark-type O_2 sensitive electrode (Hansatech Pentney, Norfolk, England). We first calibrated the system using a N_2 saturated solution to establish a zero O_2 level within the reaction chamber prior to experimental measurements. The initial series of reactions contained 1 nM of the tested antioxidant and were carried out at RT in solutions containing 50 mM tris(hydroxymethyl)amino-methane (Tris) buffered to pH 7.2. The buffer and solution containing the coordination complex were initially mixed in the reaction chamber for 20 s to establish a baseline, after which H_2O_2 was injected to initiate the evolution of O_2 .³⁴ Subsequently, we measured the initial rates of reactions between 10 mM H_2O_2 and 1.0–100 nM of the catalysts to calculate k_2 rate constants. The data were corrected to account for uncatalyzed H_2O_2 decomposition.

Peroxidase activity can also contribute to the degradation of H_2O_2 , representing a means to consume H_2O_2 without generating O_2 . This potential reactivity was evaluated by monitoring the abilities of the antioxidants to promote the reaction between H_2O_2 and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS); this reaction generates the radical cation ABTS^+ ($\epsilon_{417} = 34.7 \text{ mM}^{-1} \text{ cm}^{-1}$).³⁵ One series of reactions were run in RT 50 mM acetate solution buffered to pH 5.0, with reaction concentrations of 10 mM ABTS, 0.1 mM coordination complex, and 1–500 mM H_2O_2 . Another series of reactions were run in RT 50 mM acetate solution buffered to pH 5.0, with reaction concentrations of 10 mM H_2O_2 , 0.1 mM coordination complex, and 1–500 mM ABTS. The conversion of ABTS to its radical cation was followed using a Shimadzu UV-1601 spectrophotometer (Columbia, MD). The following equation is used to convert the absorbance data into initial rates ($v_o/[M]_T$):

$$\frac{v_o}{[M]_T} = \frac{\Delta \text{Abs (at 417 nm for 60 s)}}{60 \text{ s} \times 100 \text{ } \mu\text{M} \times 0.0347 \text{ } \mu\text{M}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}}$$

where 100 μM is concentration of the catalyst and 0.0347 $\mu\text{M}^{-1} \text{ cm}^{-1}$ is the molar absorptivity of $\text{ABTS}^{+\cdot}$ at 417 nm.

Syntheses

(1-(2,5-Dihydroxybenzyl)-8-(2,5-dihydroxybenzylalkoxide)-1,4,8,11-tetraazacyclotetradecane)zinc(II) triflate ($[\text{Zn}^{\text{II}}(\text{H}_3\text{-qp4})](\text{OTf})$, **3**). $\text{H}_4\text{qp4}$ (500 mg, 1.12 mmol) and $\text{Zn}^{\text{II}}(\text{OTf})_2$ (415 mg, 1.12 mmol) were dissolved in 5 mL of dry MeCN and then stirred at 60 °C for 24 h. The slow addition of ether to the MeCN solution deposited the product as a colorless powder that could be isolated by filtration (703 mg, 88% yield). Crystals suitable for single crystal X-ray diffraction were grown by layer diffusion of ether to a saturated solution of the crude product in MeOH. Optical spectroscopy (H_2O , 294 K): 299 nm ($\epsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$). IR (cm^{-1}): 3403 (s), 3260 (s), 3055 (s), 2988 (w), 2887 (w), 2724 (m),

1607 (w), 1508 (s), 1432 (w), 1375 (m), 1300 (m), 1247 (w), 1217 (w), 1196 (w), 1147 (w), 1115 (w), 1101 (m), 1077 (m), 1061 (s), 1021 (s), 1004 (w), 961 (m), 939 (w), 914 (m), 894 (m), 869 (w), 830 (m), 781 (m), 759 (m), 630 (s), 567 (w). ^1H NMR (500 MHz, CD_3OD , 298 K): δ 6.64–6.75 (m, 6H), 4.57 (s, 3H), 2.68 (d, $J = 25 \text{ Hz}$, 6), 2.28 (s, 2H), 2.03 (s, 2H), 1.34–1.35 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD , 298 K): δ 151.83, 149.78, 149.00, 122.99, 120.48, 119.96, 119.84, 119.45, 117.92, 117.69, 117.56, 117.16, 116.99, 58.54, 54.84, 54.54, 53.80, 53.67, 53.05, 51.91, 51.79, 50.99, 25.82, 25.78, 24.23. MS (ESI): calcd for $[\text{Zn}(\text{H}_3\text{qp4})]^+$, m/z 507.1944; found, m/z 507.1941. Elemental analysis (powder) calcd for $\text{C}_{25}\text{H}_{35}\text{N}_4\text{O}_7\text{F}_3\text{S}_1\text{Zn} \cdot 0.5(\text{C}_2\text{-H}_5)_2\text{O} \cdot 0.5 \text{ MeCN}$ (powder): C, 46.99%; H, 5.84%; N, 8.81%. Found: C, 46.52%; H, 5.99%; N, 8.81%.

Results

Synthesis and structural characterization of $[\text{Zn}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$

$\text{Mn}(\text{II})$ and $\text{Fe}(\text{II})$ complexes with $\text{H}_4\text{qp4}$ were previously reported.^{15,16} The isolated compounds, $[\text{Mn}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$ (**1**) and $[\text{Fe}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$ (**2**), feature the singly deprotonated ligand $\text{H}_3\text{qp4}^-$; we believe that the residual non-complexed ligand deprotonates the metal-bound $\text{H}_4\text{qp4}$. The complexation of $\text{Zn}(\text{II})$ to $\text{H}_4\text{qp4}$ similarly yields a $\text{H}_3\text{qp4}^-$ complex: $[\text{Zn}^{\text{II}}(\text{H}_3\text{-qp4})](\text{OTf})$ (**3**). As with **1** and **2**, the synthesis of **3** required us to heat the mixture of ligand and metal salt for a prolonged period of time (24 h) to maximize complexation of the metal ion. Complex **3** was characterized by NMR, IR, MS, and UV/vis (Fig. S1–S5†).

The redox capabilities of **3** were initially assessed using cyclic voltammetry (CV, Fig. S6†). In phosphate solution buffered to pH 7.2, we observe one irreversible redox event with an $E_{\text{pa}} = 225 \text{ mV}$ and an $E_{\text{pc}} = -11 \text{ mV}$ (vs. Ag/AgCl). This resembles irreversible CV features seen for both **1** and **2**. The redox event for the $\text{Mn}(\text{II})$ complex has an $E_{\text{pa}} = 240 \text{ mV}$ and an $E_{\text{pc}} = -20 \text{ mV}$;¹⁵ whereas, that for the $\text{Fe}(\text{II})$ compound has an $E_{\text{pa}} = 240 \text{ mV}$ and an $E_{\text{pc}} = -60 \text{ mV}$. A smaller feature is observed with $E_{\text{pc}} = 5 \text{ mV}$; this may be attributable to acid/base behavior for either the quinol or the semiquinone. A similar feature was observed for **2**.¹⁶

We crystallized **3** from MeOH/ether and structurally characterized the complex through single crystal X-ray diffraction (Fig. 1 and Table S1†). The $\text{H}_3\text{qp3}^-$ ligand coordinates to the $\text{Zn}(\text{II})$ through five out of its six possible donor atoms, with the neutral quinol not directly binding to the metal center. The coordination geometry of the N_4O donors around the $\text{Zn}(\text{II})$ is best described as a distorted trigonal bipyramidal, with a τ_5 value of 0.70.³⁶ The pendent quinol may hydrogen bond to the outer-sphere triflate; the distance between O(3) and O(6) is 2.72 Å.

Aqueous solution characterization of $[\text{Zn}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$

We investigated the stability and speciation of **3** in water using potentiometric pH titrations (Fig. S7†). As the pH of a 1:1 mixture of $\text{Zn}^{\text{II}}(\text{OTf})_2$ and $\text{H}_4\text{qp4}$ was increased from 2.5 to 10, we observe two ionization events consistent with pK_a values of 6.16 and 9.71 (Table S2†). We assign these to the deprotonation





Fig. 1 ORTEP representation of the structure of $[\text{Zn}^{\text{II}}(\text{H}_3\text{qp4})]^+$. The triflate counteranion and all H atoms are omitted for clarity. All ellipsoids are drawn at 50% probability. Full crystallographic data are provided in the ESI and in the Cambridge Structural Database (number 2173563†).

of the quinols. Although the first value is consistent with other $\text{M}(\text{II})$ -quinol $\text{p}K_{\text{a}}$ values that we have measured,^{6,8,9,15} the second value is much higher and more consistent with a metal-free quinol or phenol. The solid-state structure featuring a penta-coordinate metal center (Fig. 1) may therefore be retained in water. Despite the one fewer chelating group, the $\text{Zn}(\text{II})$ - $\text{H}_3\text{qp4}$ complex appears to be extremely stable against metal ion dissociation, with a $\log K_{\text{ML}} = 41.1$; this value is higher than the analogous values for **1** and **2**.^{15,16}

Superoxide dismutase mimicry

Compounds **1**, **2**, and **3** were initially screened using the hypoxanthine/xanthine oxidase/lucigenin assay (Fig. 2).^{28,29} By this measure, both **1** and **2** initially appeared to be capable of catalyzing superoxide dismutation. The $\text{Mn}(\text{II})$ complex **1** is most active, with an IC_{50} value of 15 nM. The $\text{Fe}(\text{II})$ complex **2** also has above-baseline activity, with an IC_{50} value of 21 nM. These IC_{50} values are similar to those measured for $[\text{Mn}^{\text{II}}(\text{H}_2\text{-qp1})(\text{MeCN})]^{2+}$ (**4**) and $[\text{Mn}^{\text{II}}(\text{H}_4\text{qp2})\text{Br}_2]$ (**5**, Scheme 1),⁶ which were subsequently confirmed to be functional SOD mimics *via* analysis of the direct reactions between the manganese compounds and KO_2 .⁸ Surprisingly, the assay data suggest that **1** and **2** could outperform the copper/zinc superoxide dismutase that served as the positive control ($\text{IC}_{50} = 40$ nM).³⁰ Compound **3** is essentially inactive, with an IC_{50} of 515 nM.

The aforementioned assay is somewhat notorious for providing misleading results due to possible side reactions between its components.^{31,37–43} In the case of **4** and **5**, the assay predicted similar activities, but later stopped-flow kinetics analysis of the direct reactions between the $\text{Mn}(\text{II})$ compounds and KO_2 indicated that the $\text{H}_2\text{qp1}$ complex was much more active than the $\text{H}_4\text{qp2}$ complex in HEPES solutions.⁸ Further, both compounds had k_{cat} values that were much lower than those predicted by the assay. Complexes **1**, **2**, and **3** were studied in 60 mM MOPS buffered to either pH 7.4 or pH 7.8 and 50 mM phosphate buffered to pH 7.4 (Table 1). The stopped-flow kinetics data indicate that **1** is significantly less active than either **4** or **5** in either a sulfonate-containing buffer or phosphate (Fig. S8†). As with **4** and many other manganese-



Fig. 2 Superoxide scavenging effects of **1** (black), **2** (pink), and **3** (green). $\text{O}_2^{\cdot-}$ was generated from the reaction between hypoxanthine and xanthine oxidase reaction and detected using the chemiluminescent probe lucigenin. All reactions were carried out in pH 7.2 phosphate buffered saline (PBS) (50 mM phosphate). Data for the various concentrations of the three coordination complexes are expressed as percentages of luminescence in the presence of vehicle.

Table 1 Catalytic rate constants, k_{cat} ($\text{M}^{-1} \text{s}^{-1}$), measured by stopped-flow kinetics for the direct reactions of **1**, **2**, and **3** with superoxide

Buffer, pH	1	2	3	4 ^a	5 ^a
60 mM MOPS/HEPES, 7.4	6.0×10^6	N.A.	N.A.	9.7×10^7	1.2×10^7
60 mM MOPS, 7.8	4.5×10^6	N.A.	N.A.	N.D.	N.D.
50 mM phosphate, 7.4	2.9×10^6	N.A.	N.A.	8.0×10^6	1.0×10^7

^a Data from ref. 8; the first-row data for **4** and **5** were collected in HEPES, rather than MOPS.

containing SOD mimics studied by ourselves,^{8,31,44} complex **1** is less catalytically active in phosphate buffer. The iron and zinc complexes, **2** and **3**, have no noticeable impact on the rate of decomposition of $\text{O}_2^{\cdot-}$ when analyzed by stopped-flow kinetics. The free $\text{H}_4\text{qp4}$ ligand is likewise inactive as a catalyst.

The obtained results further demonstrate that indirect assays often cannot even qualitatively distinguish between SOD active and inactive compounds. Instead of being more active than the SOD used as the positive control, **2** does not display any catalytic SOD activity at all and that for **1** is rather low (Table 1).

Although **3** is not an effective SOD mimic, it does react with KO_2 , as assessed by EPR, UV/vis, and MS analysis (Fig. S9–S11†). The EPR data show a weak signal at $g = 2$ at 30 s that completely disappears within 15 min. We do not believe that the EPR feature corresponds to residual $\text{O}_2^{\cdot-}$ since the oxidant should be consumed to levels below the limit of EPR detection within 1 s, even in the absence of a catalyst.⁹ The g value of the signal and its short lifetime are consistent with organic radicals similar to what we observed for previous $\text{Zn}(\text{II})$ -quinoxyl radical species.^{9,10} A $\text{Zn}(\text{II})$ -semiquinone species observed with the $\text{H}_4\text{qp2}$ ligand, for instance, persists to detectable levels for over 45 min.¹⁰ The UV/vis data indicate that the complex is initially deprotonated by KO_2 , which is a base as well as an oxidant, and



then slowly oxidized to Zn(II)-*para*-quinone species over 90 s. The MS data indicate that the reaction with $O_2^{\cdot-}$ degrades the complex (Fig. S11†). Although **3** does react with $O_2^{\cdot-}$, this activity is not enough to noticeably accelerate superoxide degradation beyond the rate observed for the uncatalyzed reaction.

Catalase activity

Previously, **1** and **2** had been found to react with excess H_2O_2 in a manner consistent with catalase activity.^{15,16} We further investigated this potential catalysis by monitoring the reactions of these two compounds, **3**, and metal-free H_4qp4 with H_2O_2 using oxygraphy and UV/vis.

The metal-free H_4qp4 ligand was unable to catalyze the degradation of H_2O_2 . Each coordination complex, conversely, catalyzes the decomposition of H_2O_2 . In all three cases, the reactivity is consistent with Michaelis–Menten kinetics with clear saturation behavior observed at high concentrations of H_2O_2 (Fig. 3). Oxygraphic measurements confirm that O_2 is being evolved; these data were fitted to the re-arranged Michaelis–Menten equation, which has parameters k_{cat} and k_{cat}/K_M (Tables 2 and S3†). The iron complex **2** appears to be slightly more active than the other two, but the individual rate constants do not vary much within this series.

Full details regarding the models used to fit the data for the three compounds are provided in Table S3 in the ESI.† The listed errors represent one standard deviation.

Second-order rate constants (k_2) were calculated by measuring the initial rates of O_2 production *via* oxygraphy with catalyst concentrations ranging from 1.0 to 100 nM (Fig. 4). The k_2 values are much lower than the k_{cat}/K_M values obtained from the Michaelis–Menten models but more accurately represent the activities of the catalysts.²² We also measured turnover numbers (TON) by quantifying the total O_2 made over time



Fig. 3 Plots of $v_o/[M]$ vs. the concentration of H_2O_2 , where $[M]$ is the concentration of the tested H_4qp4 complex: **1** (black), **2** (pink), **3** (green). The v_o corresponds to the formation of O_2 , which was measured through oxygraphy. All reactions were performed in 25 °C 50 mM Tris buffered to pH 7.2. 1 nM of each coordination complex was present as a catalyst. Each shown data point is the average of at least five independent runs. Further details regarding the models used to fit the data can be found in Table S3.†

(Fig. S12†). This parameter depends on both the robustness and activity of the catalyst and is arguably the most accurate measure of the practicality of a catalase mimic. Due to the high activity of the three complexes, we needed to perform these measurements with lower concentrations of the coordination complexes; otherwise, the O_2 evolution is too fast to monitor *via* oxygraphy. Mohammed and Gross encountered similar issues in their studies of the Fe(III) complex with the fluorinated corrole.²⁷ Although the conversions of H_2O_2 to O_2 are relatively low for these measurements, the amount of generated O_2 (A) still greatly exceeds that of the uncatalyzed reaction and (B) is highly replicable between experiments. We obtained similar TON and 10-fold greater conversions when the initial concentration of H_2O_2 was 1.0 mM rather than 10 mM (Fig. S12†). As assessed by both the initial rates analysis and the overall O_2 production, **2** clearly outperforms its manganese and zinc analogs.

The activity was qualitatively confirmed with parallel experiments that monitored the disappearance of H_2O_2 (Fig. S13 and Table S4†). The rate constants from these experiments are much less reliable than those calculated from O_2 production since all three complexes absorb strongly at the monitored 240 nm wavelength, and this technique generally tends to overestimate the activities of catalase mimics.²² Nonetheless, the activities of the complexes follow the same general order, with **2** and **3** being the most and least effective catalase mimics, respectively.

Peroxidase activity

The three H_4qp4 complexes were also assessed as peroxidase mimics using an established protocol that assesses the ability of a compound to catalyze the reaction between H_2O_2 and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS).³⁵ Both **1** and **2** appear to catalyze the reaction, as evidenced by the formation of $ABTS^+$. Complex **3**, conversely, shows no activity. Above 20 mM H_2O_2 , the reactivity seen for **1** and **2** displays no dependence on the concentration of H_2O_2 , suggesting that the activation of the bound H_2O_2 , rather than H_2O_2 binding itself, has become rate-determining (Fig. S14†). The initial rates scale with $[ABTS]_o$. Using these data, we calculated third-order rate constants of $0.41 \pm 0.02 M^{-2} s^{-1}$ and $33.8 \pm 1.2 M^{-2} s^{-1}$ for **1** and **2**, respectively. At 10 mM H_2O_2 and 10 mM ABTS, the peroxidase activity ($k_{obs} = 4.1 \times 10^{-5} s^{-1}$ for **1**, 3.4×10^{-3} for **2**) is negligible compared to the catalase activity ($k_{obs} = 13 s^{-1}$ for **1**, $17 s^{-1}$ for **2**).

Mechanistic studies

Previous UV/vis and EPR analyses of the reactions between **1** and **2** and excess H_2O_2 indicated that the quinol portions of the ligands oxidize to *para*-quinones after a variable induction period and that the Fe(II) in **2** eventually oxidizes to Fe(III).^{15,16} Magnetic susceptibility measurements suggest that **2** is mostly oxidized to high-spin Fe(III) species at RT, but EPR measurements taken at 77 K show a small amount of a low-spin signal ($g = 2.55, 2.27, 1.99$), possibly indicating that some of the Fe(III) undergoes a spin-crossover upon cooling.¹⁶

We further analyzed the reactions between H_2O_2 and the three H_4qp4 complexes by EPR, with an emphasis on collecting



Table 2 Michaelis–Menten rate constants, k_2 rate constants, and turnover numbers (TON) calculated from oxygraphic data

Complex	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)	K_M (M)	k_2 ($\text{M}^{-1} \text{s}^{-1}$)	TON
1 (Mn)	1.4×10^3	3.2×10^4	4.6×10^{-2}	$1.5 (\pm 0.1) \times 10^3$	80 (± 3)
2 (Fe)	2.2×10^3	5.2×10^4	4.2×10^{-2}	$2.2 (\pm 0.1) \times 10^3$	130 (± 1)
3 (Zn)	1.3×10^3	1.1×10^4	1.2×10^{-1}	$1.2 (\pm 0.1) \times 10^3$	51 (± 1)

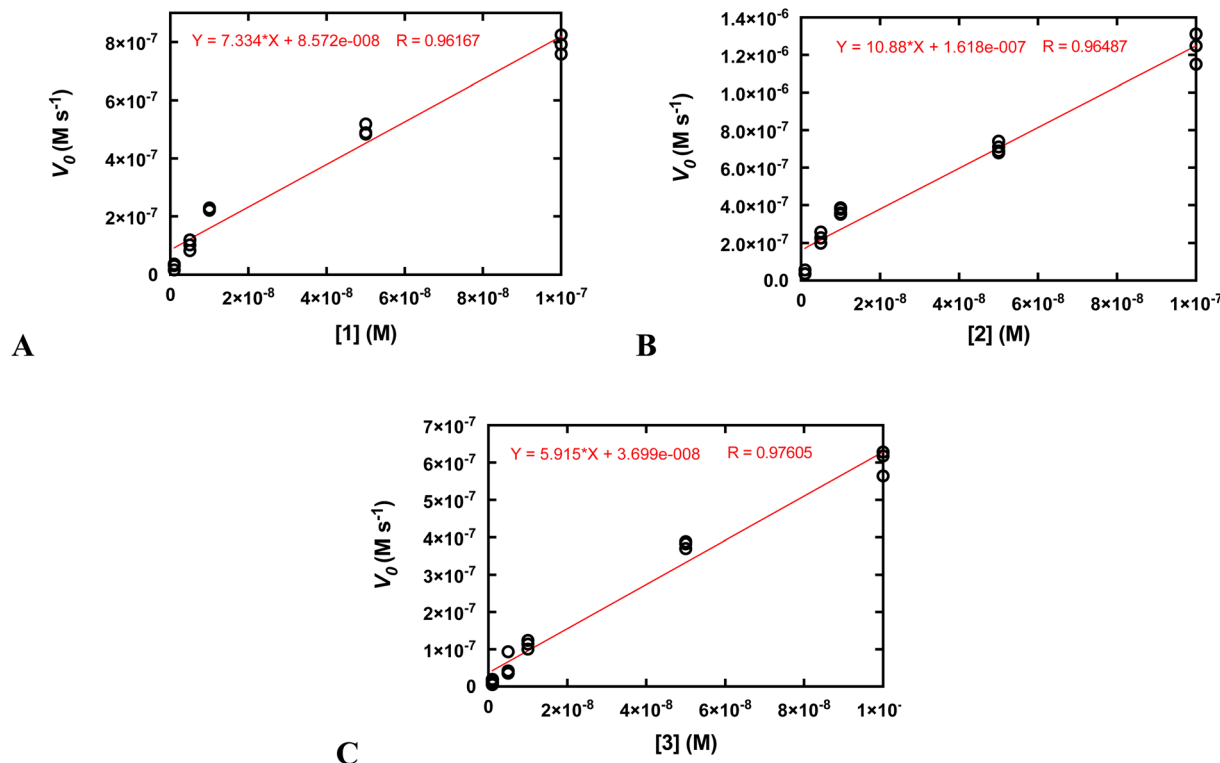


Fig. 4 Determination of k_2 from plots of the initial rates (v_0) vs. concentration of catalyst. All reactions were run at 25 °C in 50 mM Tris buffered to pH 7.2. The initial concentration of H_2O_2 was 10.0 mM for all reactions. The reaction was run at least three times at each catalyst concentration. The k_2 values on Table 2 were calculated by dividing the slopes of the shown linear fits by the concentration of H_2O_2 (0.010 M) and multiplying by two to account for the reaction stoichiometry (2 equiv. of H_2O_2 are consumed per equiv. of O_2 produced). (A) Data for 1. (B) Data for 2. (C) Data for 3.

data at an earlier time point that would coincide with higher catalase activity. EPR analysis suggests that some of the Mn(II) and Fe(II) in 1 and 2 has been oxidized 30 s after the reactions begin (Fig. S15[†]). With 1, the signal intensity for the Mn(II) decreases by approximately 10%. When 2 is oxidized by H_2O_2 , a weak signal develops with $g = 2.04$, 1.99. Relative to the intensity of the Fe(III) end-products,¹⁶ approximately 25% of the iron has been oxidized to Fe(III) by 30 s. The Fe(III) feature is distinct from the high-spin Fe(III) product that was previously observed as the major end-product of the reaction¹⁶ and is instead consistent with a low-spin Fe(III) species. With 3, we observe a weak signal with $g = 2$. The intensity of the signal is barely above the noise level, however, and it is ambiguous whether this can be attributed to a species on the main catalytic cycle.

UV/vis analysis suggests that the quinols are oxidized during the reaction between 3 and H_2O_2 (Fig. 5). With an initial concentration of 0.6 mM H_2O_2 , the 299 nm band attributable to

the quinols almost completely vanishes by 15 min. The rate of quinol oxidation is approximately as fast as that observed for 1, which likewise lacks obfuscating charge transfer bands, under the same conditions.¹⁵ When the initial concentration of H_2O_2 is 10 mM, the rate of quinol oxidation slows relative to that seen at 0.6 mM H_2O_2 , as was previously seen with 1 and 2.^{15,16} Such behavior is in agreement with the catalase activity of these complexes. An initially generated catalytically active species can either oxidize H_2O_2 or undergo self-oxidation (e.g. oxidation of the quinols, which is monitored in Fig. 5). Therefore, the more H_2O_2 that is initially present in the solution, the longer catalytic H_2O_2 decomposition proceeds and the longer the quinol groups of the ligand remain in their original reduced states. This explains why the quinols are oxidized more slowly with 10 mM H_2O_2 . When there is a lower concentration of H_2O_2 , the initially generated active species does not have enough substrate (H_2O_2) with which to react and more readily attacks the quinol moieties



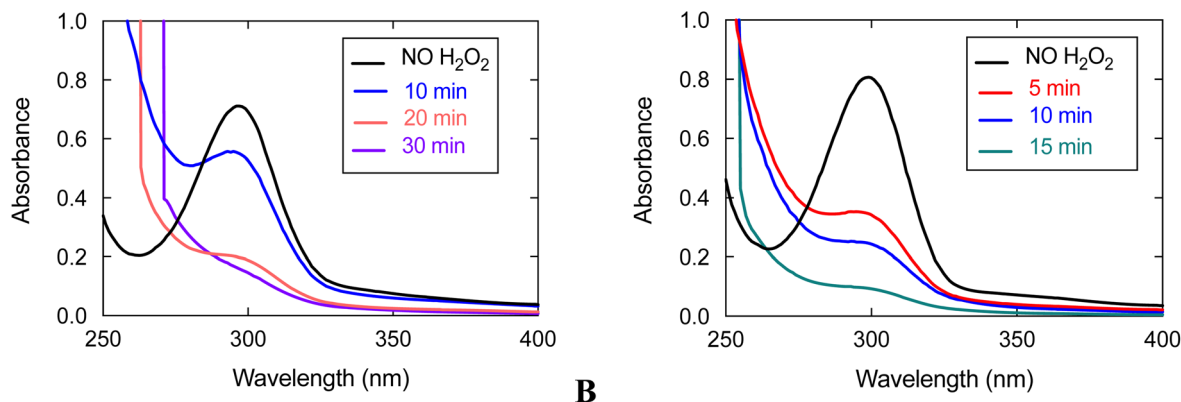


Fig. 5 UV/vis spectra of reactions between **3** and H_2O_2 . (A) Data for the reaction between 0.1 mM **3** and 10 mM H_2O_2 . (B) Data for the reaction between 0.1 mM **3** and 0.60 mM H_2O_2 . All data were taken in 50 mM HEPES buffered to pH 7.00 at 298 K with a 1.0 cm cuvette.

of the ligand, thereby resulting in faster ligand oxidation shown in Fig. 5.

With an initial concentration of 10 mM H_2O_2 , however, the quinols in **3** oxidize much more quickly than those in **1** under the same conditions. The quinols in **3** appear to be almost entirely consumed by 30 min; whereas, ligand oxidation does not occur to a noticeable extent for **1** by 30 min. The results suggest that the redox-active ligand is more heavily involved in H_2O_2 degradation for **3** than it is for **1**. Since **3** has lower catalase activity than **1**, it is more susceptible to degradation than the more active **1** in the presence of the same concentration of H_2O_2 .

We reacted each $\text{H}_4\text{qp4}$ complex with equimolar amounts of H_2O_2 in attempts to identify intermediates that might precede quinol oxidation by UV/vis (e.g. M-OOH species). In each case, however, the quinol oxidation occurs immediately, and no new features were observed (Fig. S16†). The final spectra for **1** and **2** resemble those observed for the end-products of previous reactions with large excesses of the terminal oxidant.^{15,16}

MS studies of reactions between 10 mM H_2O_2 and the $\text{H}_4\text{qp4}$ complexes in MeCN further suggest that ligand-derived redox is more prevalent for **3** than it is for the two $\text{H}_4\text{qp4}$ complexes with redox-active metals. The data for **1** and **2** at 30 s look identical to those in solutions that lack H_2O_2 ,^{15,16} the major m/z peaks are consistent with $[\text{Mn}^{\text{III}}(\text{H}_2\text{qp4})]^+$ and $[\text{Fe}^{\text{III}}(\text{H}_2\text{qp4})]^+$, respectively (Fig. S17 and S18†). Without H_2O_2 , the Mn(II) and Fe(II) appear to oxidize spontaneously under MS ionizing conditions. The data are inconsistent with M(II) or M(III) complexes with oxidized forms of the ligand, leading us to conclude that the ligand exists as the doubly deprotonated $\text{H}_2\text{qp4}^{2-}$ rather than the monoquinol/mono-*para*-quinone $\text{H}_2\text{qp4}$. When we react **3** and 10 mM H_2O_2 , however, we observe a much different set of m/z peaks that are consistent with ligand oxidation (Fig. S19 and S20†). Specifically, we detect a m/z peak at 505.1790 at 30 s that is consistent with a Zn(II) complex with the monoquinolate/mono-*para*-quinone form of the ligand (calculated $m/z = 505.1794$). Further, we observe peaks that are consistent with the addition of one oxygen atom to the mono-*para*-quinone complex ($[\text{Zn}(\text{Hqp4} + \text{O})]^+$) with $m/z = 521.1736$ (calculated $m/z = 521.1743$) and with the addition of two oxygen atoms to the

diquinol complex ($[\text{Zn}(\text{H}_3\text{qp4} + 2\text{O})]^+$) with $m/z = 539.1395$ (calculated $m/z = 539.1848$). After 60 s, oxygenated products appear to become more prominent (Fig. S21†). We analyzed longer-term reactions between **1** and **2** with larger excess of H_2O_2 and likewise found evidence of ligand oxidation beyond the quinol-to-*para*-quinone conversion. With **1**, we detect m/z peaks consistent with the loss of quinol groups (Fig. S22†). With **2**, new m/z peaks are observed, but these are more difficult to assign to specific oxidation products (Fig. S23†).

Discussion

The manganese and iron complexes with 1,8-bis(2,5-dihydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane ($\text{H}_4\text{qp4}$) were previously found to act as T_1 -weighted MRI contrast agent sensors for H_2O_2 .^{15,16} The responses, which rely either wholly or partly on the oxidation of the quinolic portions of the ligand, were found to both display an induction period and be noticeably slower with larger excesses of H_2O_2 . This initially counter-intuitive observation led us to speculate that both $[\text{Mn}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$ (**1**) and $[\text{Fe}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$ (**2**) were proceeding through higher-valent metal species that could either self-oxidize to the activated sensor or catalyze the decomposition of the excess H_2O_2 (Scheme 3).

Given that other quinol-containing ligands had been found to allow the redox-inactive metal ion Zn(II) to catalyze the dismutation of superoxide,^{9,10} we also prepared and tested the reactivity of a Zn(II) complex with $\text{H}_4\text{qp4}$, $[\text{Zn}^{\text{II}}(\text{H}_3\text{qp4})](\text{OTf})$ (**3**). The synthesis of the Zn(II) complex was identical in most aspects to those used to prepare both **1** and **2**, and as with these syntheses, a complex with singly deprotonated ligand ($\text{H}_3\text{qp4}^-$) was the isolated product.^{15,16} Unlike the manganese and iron systems, we were able to obtain a crystal structure for the divalent metal. The $\text{H}_3\text{qp4}^-$ ligand does not fully coordinate to the metal center (Fig. 1), which may suggest that the Zn(II) is too small to be fully accommodated by the ligand pocket. Only one of the quinols is coordinated in the crystal structure, and it appears that it remains unassociated with the metal center in aqueous solution since the second measured pK_a of 9.71 is only slightly lower than the value of ~ 10 expected for a non-



complexes to function in water would facilitate the application of either these complexes or more highly performing derivatives towards the clinical treatment of oxidative stress.

Despite the lesser peroxidase activities, complexes 1–3 do eventually degrade when exposed to exceedingly high concentrations of H_2O_2 (Fig. S21–S23[†]). This limits the TON, as evidenced by the low conversions (0.10–0.22%) observed in Fig. S12;[†] in these experiments, 0.1 μM of each complex reacts with 10 mM H_2O_2 . In light of this result, we reviewed the conditions used to study the MRI properties of 1 and 2 and determined that the 0.1–1.0 mM concentrations for the MRI studies were sufficient to completely degrade the 10 mM of H_2O_2 used to activate the sensors.^{15,16} We will also note that we did not observe any ligand oxidation beyond the quinol-to-*para*-quinone conversion in these prior studies.

That 1, 2, and 3 catalyze H_2O_2 dismutation at similar rates led us to initially hypothesize that the three catalysts mainly rely on a common mechanistic cycle that does not feature redox at the metal center. EPR analysis of the reactions between H_2O_2 and 1 and 2 at 30 s, however, reveals that the metals in these complexes do noticeably oxidize, with the Mn(II) signal diminishing and a novel low-spin Fe(III) signal appearing, respectively. The low-spin Fe(III) signal seen for 2 could potentially correspond to an Fe(III)–OOH species,⁵² but other species could give rise to a similar signal. We attempted to determine whether any Fe(III)–OOH species were present using resonance Raman spectroscopy but were regrettably unable to locate any diagnostic O–O or Fe–O stretches. Our prior analysis of the reactions between H_2O_2 and 1 and 2 suggested that the quinols in the $\text{H}_4\text{qp4}$ ligand do not start to convert to *para*-quinones until most of the H_2O_2 has been depleted.^{15,16} With a stoichiometric amount of H_2O_2 , ligand oxidation occurs immediately for both 1 and 2 (Fig. S16[†]).

UV/vis and MS analysis of the reactions between 3 and H_2O_2 , conversely, demonstrate that one of the quinols oxidizes to a *para*-quinone early in its reaction with a large excess of H_2O_2 (Fig. 5 and S19[†]). Additionally, we observe a m/z feature at 539.1396; the mass and charge are consistent with a Zn(II)–OOH complex with the mono-*para*-quinone form of the $\text{H}_4\text{qp4}$ ligand, $\text{H}_2\text{qp4}$. We caution that the data could also correspond to other species, such as a Zn(II)–OH complex with an oxygenated ligand and that we were likewise unsuccessful in our attempts to visualize a Zn(II)–OOH species with resonance Raman spectroscopy. The ambiguity in the MS data would not be resolvable by ^{18}O -labeling studies. We do not observe any MS data consistent with a diquinone (qp4) species. Based on these results, we tentatively propose that the H_2O_2 dismutation for 3 proceeds through the cycle shown in Scheme 4. H_2O_2 reacts with $[\text{Zn}(\text{H}_3\text{qp4})]^+$ to yield a M(II)–OOH species (B), with subsequent intramolecular oxidation of one of the quinols to a *para*-quinone (C). The coordination of H_2O_2 to Zn(II) has been previously observed,⁵³ leading us to believe that Zn(II)–OOH species are indeed plausible intermediates. A second equiv. of H_2O_2 then coordinates the metal center as HOO^- , generating $[\text{Zn}(\text{H}_2\text{qp4})(\text{OOH})]^+$ (D), which we may be detecting by MS. The *para*-quinone can then oxidize this second equiv. of H_2O_2 . Since D appears to accumulate, this may be the rate-limiting step in the catalase activity. A previous report found that acids could catalyze the reduction of *para*-quinone to quinol,⁵⁴ and the Zn(II) in 3 may



Scheme 4 Proposed mechanism for catalase activity that avoids metal-centered redox.

do likewise in its capacity as a Lewis acid. The Zn(II) could either coordinate water and facilitate the delivery of protons to the *para*-quinone, or it could promote the reduction portion of the PCET by coordinating the *para*-quinone.

Redox-inactive metal ions, such as Al(III) and Ga(III), are capable of activating H_2O_2 ; in this chemistry, hydroxyl and hydroperoxyl radicals are released upon the coordination of multiple equiv. of the oxidant to the metal center.^{55–57} Given the observed Michaelis–Menten kinetics and lack of a second-order dependence of the rate on $[\text{H}_2\text{O}_2]$, we do not believe that the simultaneous coordination of two equiv. of H_2O_2 is required for catalase activity to proceed. Further, the inability of 3 to oxidize external substrates such as ABTS is inconsistent with the release of highly oxidizing hydroxyl and hydroperoxyl radicals.

Since extensive ligand oxidation is not observed in the early stages of catalase activity, we propose that 1 and 2 mostly catalyze H_2O_2 dismutation through a fundamentally different mechanism (Scheme 5). The initial step corresponding to the coordination of H_2O_2 to the divalent metal ion remains the same, but once B is generated, the O–O bond may break heterolytically to yield a M(IV) species (C'). Such a high-valent species would be stabilized by two factors: the presence of the anionic quinolate and the ability of the quinolate to donate an electron to the metal center to yield an isoelectronic M(III)-ligand radical. Species C' can either react with a second equiv. of H_2O_2 , removing two net hydrogen atoms to yield O_2 and a M(II)–OH₂ species (D'), or it can further oxidize the ligand to a *para*-quinone species (E). Although E is shown as a hydroxyl complex in Scheme 5, the OH group is basic enough to abstract a proton from the buffered medium, yielding the aquated species observed in our prior report on 1.¹⁵ The Fe(II) in 2 eventually is oxidized to Fe(III),¹⁶ and this may occur through a side reaction between Fe(II) species and 0.5 equiv. H_2O_2 .

That 1 and 2 have higher TON than 3 likely results from improvements to both the speed and durability of the catalyst. The k_2 rate constants for 1 and 2 are both higher than that of 3 (Table 2); this is consistent with the redox-active metals opening new pathways for H_2O_2 dismutation (Scheme 5). Catalysts 1 and 2 also appear to better resist over-oxidation. If external oxidants are initially channeled towards converting quinols to *para*-quinones, one would anticipate that catalysts that can avoid the





Scheme 5 Proposed mechanism for catalase activity with metal-centered redox.

full oxidation of the quinols (Scheme 5) would be less susceptible to alternative oxidation reactions, such as benzylic C-H activation, than catalysts that rely exclusively on the quinol/*para*-quinone redox couple (Scheme 4).

Conclusions

We find that quinol-containing ligands can substantially enhance the ability of metal ions to catalyze the dismutation of H_2O_2 . The described complexes with the macrocyclic ligand $\text{H}_4\text{qp4}$ are arguably the most active small molecule catalase mimics reported to date and display negligible competing peroxidase activity. Notably, the redox-inactive Zn(II) activates the ligand for redox catalysis. Despite the similar rate constants for catalase activity, the Zn(II) complex appears to react with H_2O_2 through a fundamentally different mechanism than the complexes with manganese and iron; the complexes with the redox-active metals do not rely as heavily on ligand-centered redox processes. Although similar ligands have been used in highly efficient small molecule mimics of superoxide dismutases, the $\text{H}_4\text{qp4}$ ligand does not enable metals to rapidly dismutate superoxide. Only the manganese complex displays such activity, and even this compares poorly to those of manganese complexes with other polydentate quinol-containing ligands. As such, there appear to be subtle factors that direct antioxidant behavior towards specific reactive oxygen species. The exact factors that determine substrate selectivity and the mechanisms for how catalase mimicry is accomplished remain subjects of investigation.

Data availability

Most of the data that support the findings of this study are present in the article and its ESI.† The corresponding author will provide additional data not available in these documents upon request.

Author contributions

S. K., A. F., J. O., T. A., A. J., P. R. P., and D. D. S. conducted the experiments and collected and analyzed the data. S. K., A. F., J. O., P. R. P., and D. D. S. also wrote/edited portions of the manuscript. S. K., I. I.-B., and C. R. G. conceived the idea. I. I.-B. and C. R. G. also supervised the project, provided resources, and wrote/edited the manuscript.

Conflicts of interest

There are no conflicts to declare.

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