Low Temperature Syntheses and Reactivity of Cu$_2$O$_2$ Active-Site Models

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CONSPECTUS: Nature's facility with dioxygen outmatches modern chemistry in the oxidation and oxygenation of materials and substrates for biosynthesis and cellular metabolism. The Earth's most abundant naturally occurring oxidant is—frankly—poorly understood and controlled, and thus underused. Copper-based enzyme metallocofactors are ubiquitous to the efficient consumption of dioxygen by all

domains of life. Over the last several decades, we have joined many research groups in the study of copper- and dioxygen-dependent enzymes through close investigation of synthetically derived, small-molecule active-site analogs. Simple copper-dioxygen clusters bearing structural and spectroscopic similarity to dioxygen-activating enzymes can be probed for their fundamental geometrical, electronic, and reactive properties using the tools available to inorganic and synthetic chemistry.

Our exploration of the copper-dioxygen arena has sustained product evaluation of the key dynamics and reactivity of binuclear Cu$_2$O$_2$ compounds. Almost exclusively operating at low temperatures, from −78 °C to solution characterization even at −125 °C, we have identified numerous compounds supported by simple and easily accessed, low molecular weight ligands—chiefly families of bidentate diamine chelates. We have found that by stripping away complexity in comparison to extended protein tertiary structures or sophisticated, multinucleating architectures, we can experimentally manipulate activated compounds and open pathways of reactivity toward exogenous substrates that both inform on and extend fundamental mechanisms of oxygenase enzymes.

Our recent successes have advanced understanding of the tyrosinase enzyme, and related hemocyanin and NspF, and the copper membrane monooxygenases, specifically particulate methane monooxygenase (pMMO) and ammonia monooxygenase (AMO). Tyrosinase, ubiquitously distributed throughout life, is fundamental to the copper-based oxidation of phenols and the production of chromophores by dedicated biosynthesis or incidental oxidative browning. The copper membrane monooxygenases are comparatively new entrants to the copper-dioxygen field. While pMMO mediates the synthetically tantalizing transformation of methane to methanol, AMO catalyzes the first metabolic step in deriving chemical energy from ammonia—a reaction massively represented on a global scale and a critical component of chemical homeostasis on Earth.

In this Account, we begin by introduction of the synthetic copper-dioxygen chemistry field, from techniques to the differential coordination of dioxygen with copper. Then, we describe the unambiguous self-assembly of an oxygenated tyrosinase mimic from basic constituents (copper, dioxygen, and monodentate-imidazole histidine analogs) and the resulting emergence of intrinsic reactivity, free of any influence due to the protein environment. Next, we discuss the first catalytic oxidation of phenol through a fully characterized tyrosinase mimic, derived from molecular oxygen, and its application to substrates unreactive in the native enzyme system. Finally, we detail evidence for chemical plausibility of dioxygen activation in pMMO (and AMO) through a high-valent species and the thermodynamic criteria that beg introduction of the Cu(III) state to biological redox catalysis.
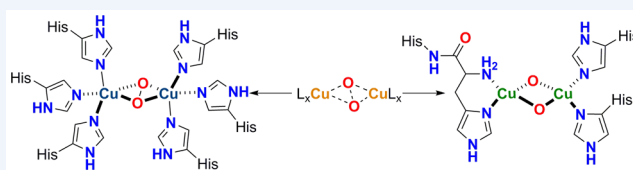
1. INTRODUCTION

Copper-based activation and metabolism of atmospheric dioxygen is fundamental to all domains of life.[1] The precise balance of binding energetics along with controlled multi-electron reactivity of dioxygen allow for the diversity of O$_2$-transport, catalytic reduction, and chemically selective transformations of biomolecules and carbon substrates identified in unicellular archaeal and bacterial biochemistry all the way to human physiology.

Our work in the arena of bioinspired inorganic chemistry has shown that elucidation and further extension of a copper-

dioxygen activation mechanism can be achieved by critical evaluation of the primary active-site coordination. Over 20 years, we have discovered dozens of characterizable copper-dioxygen compounds through the use of low molecular weight amine ligands as surrogates of nitrogenous coordination in copper-based active sites. By stripping away the complexity of biological ligation in favor of small-molecule analogs—the simpler the better[2]—a deeper understanding of the thermody-
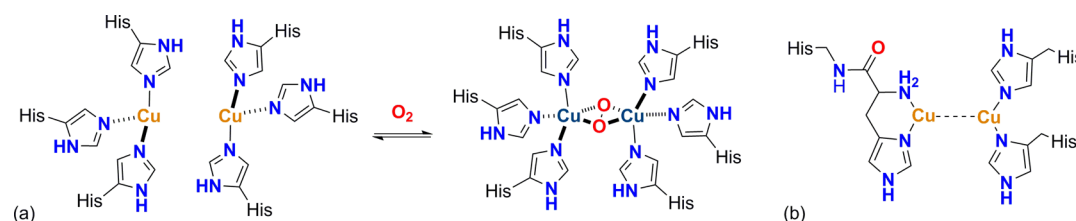
Figure 1. (a) Active site of tyrosinase. (b) Proposed binuclear active site of pMMO.
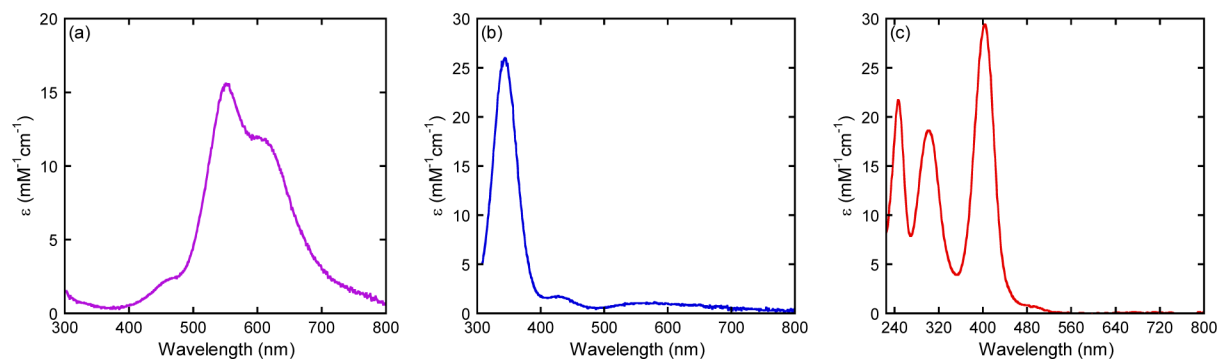

Figure 2. Optical spectra of a dimeric copper (a) Cu(II/II) trans-peroxide, (b) Cu(II/II) side-on peroxide, and (c) Cu(III/III) bis(μ-oxide).
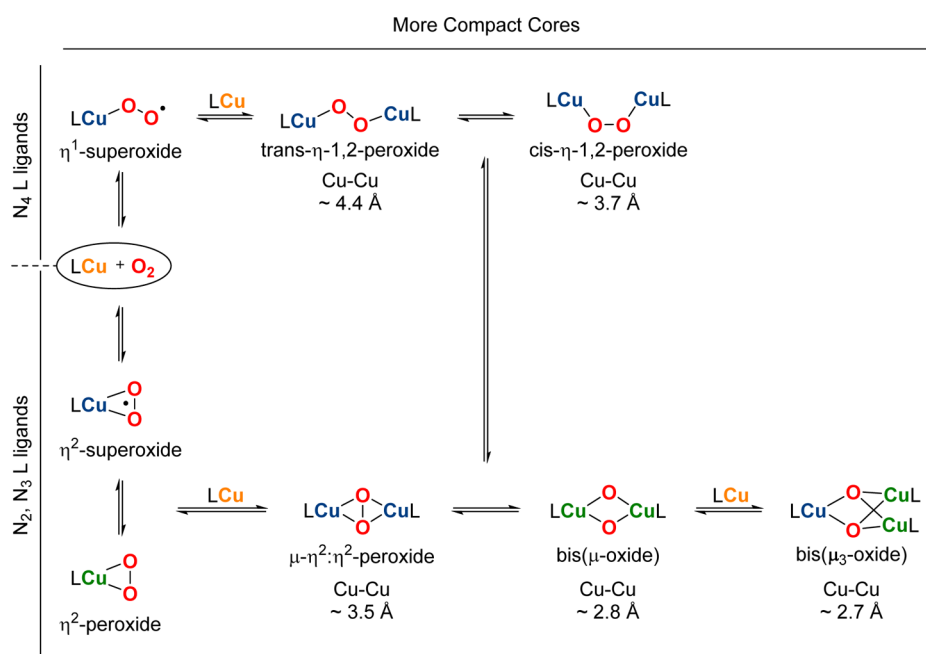

Figure 3. Reduction of dioxygen by synthetic cuprous complexes.[3]

namics of dioxygen binding modes and copper nuclearities, along with their intrinsic reactivity toward substrates, is possible with the ultimate goal of applying them to future chemistries useful to the synthetic chemist.

In recent years, we have invested in replicating biological coordination to the greatest extent possible under the constraint of minimalism in our models. In one sense, we set out to extract copper-based active sites from their protein environments. Synthetic compounds that faithfully model the *essence* of an active site are a fruitful path to systematized studies of the particular coordination effects at a copper center, which impart the *inherent* reactivity of the complexes. This Account will summarize our current efforts in modeling the copper-dioxygen dependent active sites and reactivity of tyrosinase and

copper membrane monooxygenases (most notably pMMO) largely with biologically relevant ligands (Figure 1).

2. BACKGROUND

The copper-dioxygen field is somewhat privileged among other inorganic disciplines in that the optical absorption signatures of the different types of compounds studied are remarkably diagnostic (Figure 2).[3] Absorptions are typically intense and often lie in distinct energy ranges. Optical spectroscopy coupled with Cu K edge X-ray absorption spectroscopy (XAS), extended X-ray absorption fine structure (EXAFS), resonance Raman spectroscopy, EPR, and crystallography (when possible)

allows for unambiguous characterization of complexes and determination of metrical parameters.

However, small molecule copper-dioxygen complexes are a particularly sensitive class of inorganic coordination compounds. The oxygenated Cu(II) and Cu(III) forms are almost without exception temperature sensitive; most copper-dioxygen species are only stable between −78 and −125 °C. In recent years, advances in the field have been restricted generally to extreme solution temperatures possible in 2-methyltetrahydrofuran (f.p. −136 °C), a technique first reported by our group in 2005.[4] Low solution temperatures are not an impediment. Often, adjustment of kinetics brought by reducing the thermal energy exposes unseen intermediates and provides mechanistic information unobtainable otherwise.

A great diversity of dioxygen coordination modes and copper nuclearities are accessible through selective ligand design and oxygenation conditions, discussed at length previously (Figure 3).[3] Binuclear complexes are by far the most reported. Stabilization and accumulation of mononuclear Cu(II)-superoxide or Cu(III)-peroxide compounds in homogeneous syntheses commonly necessitate careful choice of ligand denticity and steric architecture to preclude dimerization.[5,6] Of the binuclear complexes, the number of known dicopper-(III) bis(μ-oxide) complexes is largest, though it was one of the last structural motifs to be identified.[7,8] In limited cases, such species exist in a measurable, facile equilibrium with its electronic isomer, the dicopper(II) $\mu-\eta^2:\eta^2$ (side-on) peroxide,[3,9,10] which was the first copper-dioxygen binding mode observed in biology.[11−13] Tuning of the energetic relationship between the two isomers is governed nominally by two variables: the electronics and sterics of the supporting ligands— the latter being the dominant factor.[2,3] The elongated 3.5 Å Cu−Cu separation of the dicopper(II) side-on peroxide state is preferred in the context of bulkier or less donating ligands (substituted pyrazoles, pyridines, imidazoles, secondary and tertiary amines). The contracted ~2.8 Å Cu−Cu geometry of the high-valent dicopper(III) bis(μ-oxide) is biased in systems with sterically less demanding or more electronically donating ligands (methylated tertiary amines, guanidines, anionic diketiminates).

The syntheses of these complexes involve formal reduction of dioxygen by one, two, or four electrons. The electrons are derived most commonly from equivalents of Cu(I), yielding mononuclear Cu(II)-superoxides and Cu(III)-peroxides, binuclear Cu(II/II)-peroxides and Cu(III/III)-bis(μ-oxides), and trinuclear Cu(II/II/III)-bis(μ_3-oxides) (Figure 3).

In most cases, the method of oxygenation and assembly is critical to the successful formation of copper-dioxygen coordination compounds. The yields and types of compound are strictly dictated by the choice of synthetic conditions; temperature, solvent, and counteranion can be critically important. Certain complexes supported by chelates bearing amino N−H bonds or imidazole moieties are only accessible by ligand exchange reactions from stable, precursor Cu_2O_2 compounds[14,15] (vide infra). In one instance, full self-assembly, invariant to the order of addition of constituents, has effected the formation of binuclear complexes,[16] as discussed next.

3. OXYTYROSINASE SELF-ASSEMBLY

Self-assembly processes are those in which constituent elements pass through a series of individually reversible steps to a thermodynamically stable product.[17,18] Self-assembly is often applied to the generation of ordered products or structures

without external direction or biasing. Reversibility in the process introduces the ability to self-edit—to back out of intermediate structures whose energies may be locally lowered but do not represent the global minimum. Self-assembly is critically important to biology.[19] The organization of lipids into bilayers and the folding and aggregation of peptide and nucleotide polymers are synonymous with the definition of cellular life but can be observed in extracellular, abiotic environments.

With our eye toward inorganic cofactors in biology, we cite the synthetic self-assembly, now more than 40 years ago, of biomimetic $[Fe_4S_4]$ iron−sulfur cubanes, absent of any polypeptide framework, by Holm and co-workers as a pinnacle achievement in bioinorganic chemistry and the study of chemical evolution in biology.[20] More recently, researchers have characterized the high-temperature self-assemblies of asymmetric $[Mn_3CaO_4]$ clusters bearing marked similarity to the dioxygen-evolving complex of photosystem-II.[21,22] This year, Karlin has shown stepwise assembly of mixed binuclear Fe/Cu-peroxide models of heme cyctochrome-c oxidase with coordination of monodentate imidazoles on copper and the axial "distal" histidine position on iron.[23]

In our own studies of oxygenated tyrosinase, a coupled binuclear copper protein that activates dioxygen as a $\mu-\eta^2:\eta^2$-peroxide level intermediate (Figure 1a) and oxidizes phenols to o-catechols and o-quinones,[1] we have also learned to appreciate the power of self-assembly to inform on the nature of active-site design and functionality. In 2012, we demonstrated the unambiguous self-assembly of a family of functional oxytyrosinase cores stabilized by monodentate imidazoles (Figures 4−6), surrogates of the active-site histidine ligation found within the protein.[16]
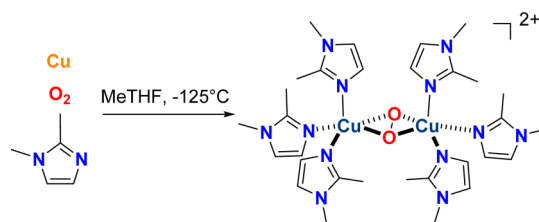

Figure 4. Low-temperature synthesis of the oxytyrosinase core.

Injection of solutions of $[Cu(MeCN)_4]SbF_6$ with 3 equiv of 1,2-dimethylimidazole into dioxygen-saturated 2-MeTHF equilibrated at −125°C quantitatively yields a dicopper(II)-$\mu-\eta^2:\eta^2$-peroxide, bearing remarkable spectroscopic congruity to the oxygenated tyrosinase. This dicopper(II) side-on peroxide exhibits an intense UV-transition at 343 nm (25 mM^{-1} cm^{-1}), a weaker visible absorption at 570 nm (1.1 mM^{-1} cm^{-1}), and a 3.57 Å Cu−Cu distance, fully consistent with the dicopper(II) side-on peroxide core identified in biology. All metrical parameters and optical features are well-reproduced by DFT and TD-DFT calculations on the proposed structure.

This peroxide compound consists of two coppers, dioxygen, and six monodentate imidazoles. The term "self-assembly" in this synthesis is demonstrated by the invariance of the formation to the order of addition of the constituents (Figure 5). These independent methods of assembly together yield no other conclusion but the inherent stability of the oxytyrosinase core, the dicopper(II) side-on peroxide, with respect to other dioxygen adducts or isomeric forms with imidazole ligation and
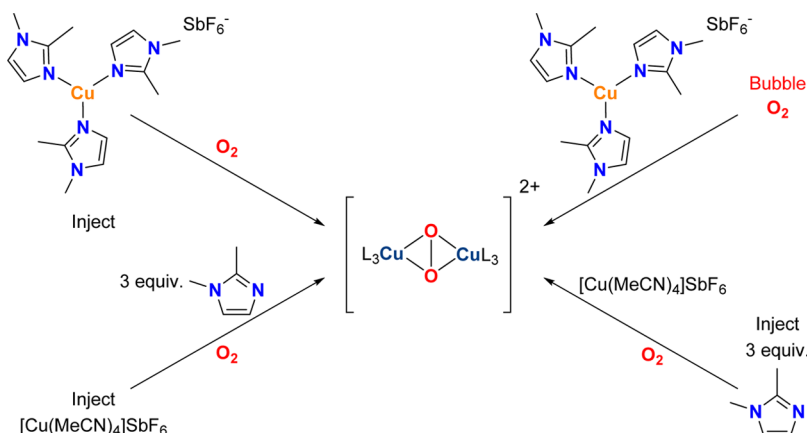
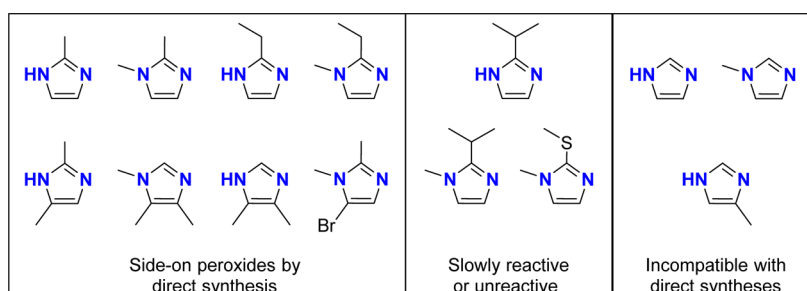**Figure 5.** Self-assembly of the synthetic oxytyrosinase core.

Figure 6. Imidazoles used in the self-assembly of the oxytyrosinase core.

absent of any direction due to a protein matrix. Thus, it is not surprising that this inherently stable, oxygenated form has been co-opted by almost every life form on the planet to effect phenol-to-quinone transformation.

Formation is nearly maximized with 3 equiv of imidazole per copper for a variety of substituted imidazoles (Figure 6). Yields of the 2:1 copper-peroxide level intermediates can be determined indirectly. Excess equiv of the strongly coordinating tris(2-dimethylaminoethyl)amine (Me$_6$Tren) can displace the monodentate imidazoles and capture the Cu$_2$O$_2$ core from the side-on peroxide. In situ optical spectroscopy shows growth of the Me$_6$Tren-stabilized μ-1,2-*trans*-peroxide (Figure 7) previously characterized by Schindler and co-workers[24] in near quantitative yields, thus benchmarking the formation of the starting side-on peroxides.

The method of completely transferring the Cu$_2$O$_2$ fragment has been particularly interesting to us, as first demonstrated by the Karlin group nearly 20 years ago.[25] In the reaction of an imidazole coordinated side-on peroxide with Me$_6$Tren, the transfer can be slow, but it is favorable and irreversible within experimental constraints. Large excess of imidazole cannot strip the multidentate tren-type ligand from copper. However, a truncated, peralkylated, bidentate analog, tetramethylpropylenediamine (TMPD), can be displaced by the nominally weakly coordinating, mondentate imidazoles. The core of previously characterized, TMPD-supported dicopper(III) bis(μ-oxide)[26] can be captured by 3 equiv of 1,2-dimethylimidazole per copper (Figure 8). The energetics of this exchange will be discussed in subsequent sections, but the feasibility of this reaction is of immediate benefit: it provides a route to structures inaccessible by the self-assembly syntheses detailed above. Minimally substituted imidazoles (1- and 5-methylimidazole; Figure 6) can effect the capture of the TMPD bis(μ-oxide) to yield
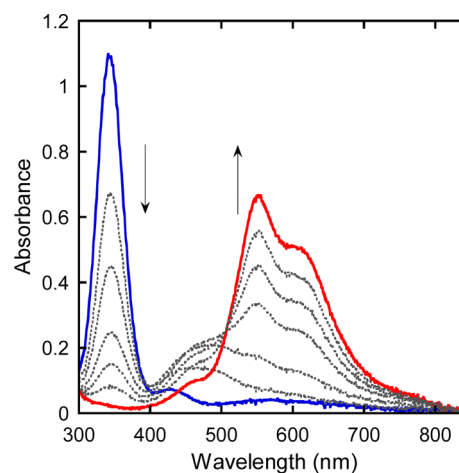

Figure 7. Core capture of oxytyrosinase analogue by Me$_6$Tren.

recognizable imidazole-bonded side-on peroxides, where formerly direct oxygenation of Cu(I) complexes were unproductive.

Finally, this family of compounds is not only structurally faithful to the oxytyrosinase active site, they are functional analog capable of regioselective hydroxylation of various *p*-substituted phenolate substrates to the corresponding *o*-catechols. This hydroxylation reactivity is noncatalytic (see section 4), as turnover to the Cu(I) state has not been possible with this system (Figure 10). One equivalent of 15-crown-5 ether per sodium phenolate is required to differentiate rates of hydroxylation between substrates. Plotting against the Hammett σ_p^+-parameter establishes a ρ value of −2.2, demonstrating rate dependence on the electron withdrawing properties of the phenolate para substituent.[4,27] Careful
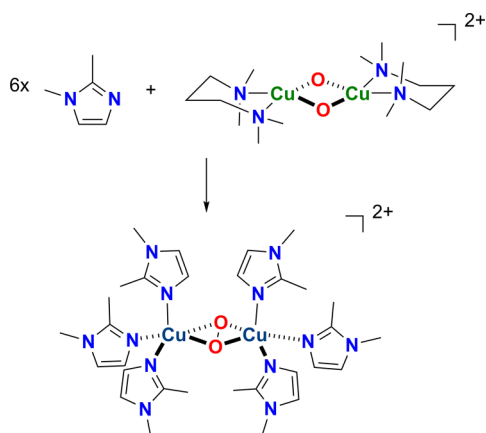
Figure 8. Quantitative core capture by 1,2-dimethylimidazole.

selection of a mixed isotope phenolate substrate allowed evaluation of an inverse *intramolecular* kinetic isotope value $KIE_{H/D} = 0.93$. This inverse value, coupled with the negative ρ-value, positively supports an electrophilic mechanism of phenolate hydroxylation, the consensus reaction path of oxytyrosinase.

We believe the value of these self-assembled oxytyrosinase mimics is not restricted to the structural or spectroscopic characterization of the enzyme active site, nor in the elucidation of finer, unresolved mechanistic subtleties of its catalysis, but bears broader significance to chemical evolution. The unmistakable gymnastics of the self-assembly synthesis speaks to the inherent stability of the $\mu-\eta^2{:}\eta^2$-peroxide in a tyrosinase-like, imidazole-based environment. Without guidance of an extended protein tertiary structure, these fundamental components assemble to innately functional species. We learn that the innate function is not conferred by particular constraints imposed on the Cu_2O_2 core by the polypeptide environment but rather is an emergent property of a thermodynamically stable species. The implication to selective chemical evolution seems clear: Nature operates through and exploits the intrinsic reactivity of stable cofactors. Biological mastery of the chemistry, through the protein matrix, lies in the control of confounding influences, such as management of reactive species, preventing disassembly of reactive complexes at operational temperatures, concentration of substrates in binding sites, and facilitating delivery of proton/electrons. Our compounds may only be studied at extreme solution temperature, where entropic terms are minimized and deleterious decay pathways are inaccessible kinetically. The protein structure serves to predispose the active-site components toward formation, effectively shifting the entropic load to the ribosome and folding processes. Tethering of the amino acid ligands to the protein matrix limits the mobility of reactive oxygen species in the active site and channels the oxidizing potential of dioxygen to productive catalysis.

4. CATALYTIC PHENOL HYDROXYLATION

Investigation of biological catalysis is inspired both by scientific curiosity of the subtleties of the natural world and the practical development of transformations useful to the synthetic chemist.[28] Whether the latter takes the form of mimicry or advantageous application of divined themes, the ultimate goal is to meet or outmatch the performance of biological systems or to expand the investigated chemistry beyond the scope of

biology, which is constrained to operate in water in a limited range of temperatures and pressures.

Close collaboration with the Herres-Pawlis group afforded us the opportunity to explore whether a synthetic analogue could in fact parallel the intimate mechanistic steps of tyrosinase catalysis.[29] Through ligand screening, a tripodal bis(3-*tert*-butyl-pyrazolyl)pyridylmethane framework was identified to afford oxygenation of Cu(I) complexes to an exceptionally stable dicopper(II) side-on peroxide (Figure 9). Only two other
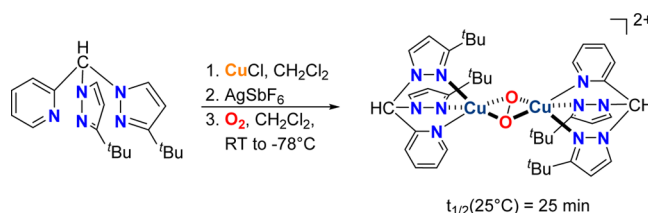

$t_{1/2}(25\,°C) = 25\ min$

Figure 9. Synthesis of a dicopper(II)-$\mu-\eta^2{:}\eta^2$-peroxide catalyst.

reported cupric dimers exhibit superior thermal stability,[30,31] though neither study indicated reactivity with exogenous substrates. Tyrosinase-like dioxygen-based oxidations have been limited to a few stoichiometric examples and only three notable reports of significant catalytic turnover.[32−35] The notable stability of the complex discussed in this section opens thermal regimes conducive to catalytic chemistry, as it is observed that multiple phenol-to-quinone turnovers are possible with simple phenols, tyrosine molecules natural to the enzyme, atypical steroid-derivative substrates, and, most importantly in our view, hydroxy-aromatics incompatible with tyrosinase.

The ligation sphere only minimally resembles that of the tyrosinase active site, though catalysis remarkable to the field is derived from a fully characterizable intermediate supported by ligation of nonbiological pyrazole and pyridine moieties. We speculate that the mixed nature of the ligating substituents permits substrate accessibility, presumably through permissible rearrangement of the coordination before phenol(ate) binding.[36]

The unanticipated stability of the peroxide adduct is, in our view, the chief criterion of catalysis in this system (Figure 10). Subordinate, but still requisite, are proton management and availability of excess dioxygen in solution.[32−34,37] However, it is the thermal energy available at higher temperatures that permits
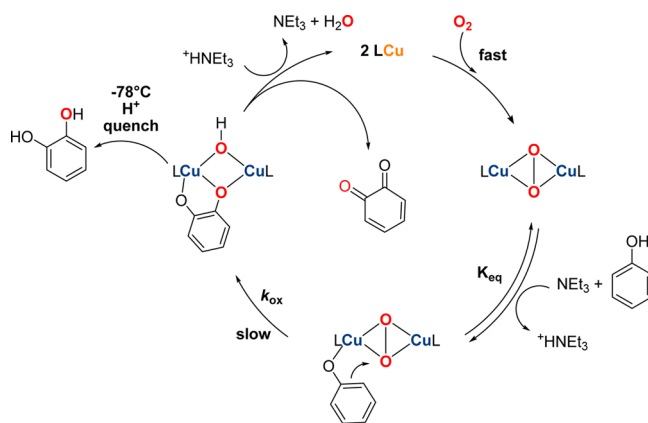

Figure 10. Catalytic mechanism of phenol oxidation by a synthetic dicopper(II) $\mu-\eta^2{:}\eta^2$-peroxide catalyst.

traversal of barriers to two-electron reduction of copper by bonded catechol. Indeed, at low temperatures, stoichiometric yields of catechol are obtained by reaction of phenolate or phenol with equivalents of base. A single equiv of catechol is expelled upon acidic quenching at low temperature. However, this intermediate yields an equivalent of *o*-quinone if warmed to 25 °C, indicating product inhibition of catalysis by catecholate, which is overcome at warmer temperatures. Multiple turnovers are possible at 25 °C with excess dioxygen and management of phenolic protons by exogenous triethylamine base.

The conclusion of this chemistry is that this synthetic system can be fitted to the catalytic mechanism of tyrosinase while opening routes to substrate transformation incompatible with the enzyme (Figure 11). The native substrate scope of
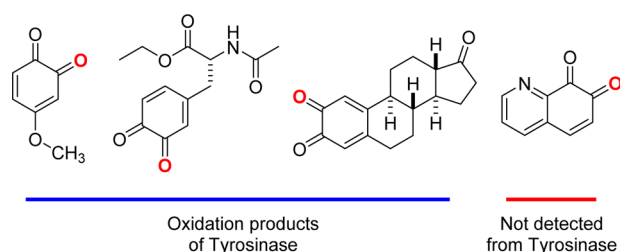

Figure 11. Quinone products of phenol oxidation by a synthetic dicopper(II)-μ-η2:η2-peroxide catalyst compared to quinones accessible from tyrosinase.

tyrosinase can be expanded to the estrogenic hormone, estrone (a viable substrate in vitro),[38] and to model substrates like 8-hydroxyquinoline, toward which tyrosinase exhibits no oxidative action. The combination of dioxygen-derived, organic-compatible, multiple-turnover catalysis of challenging substrates positions this system, derived from and informative to biological investigation, as a useful tool for selective, late-stage functionalization of an extended scope of molecules—one of the ostensibly important goals of chemical research into biological mechanisms.

5. DIOXYGEN ACTIVATION IN PMMO

The biological, chemical, and environmental scientific worlds eagerly await definitive elucidation of the methane hydroxylation reaction mediated by bacterial particulate methane monooxygenase (pMMO).[39−41] Under normal growth conditions, the predominantly expressed bacterial methane monooxygenase is pMMO, a copper- and dioxygen-dependent membrane-bound protein.[42−44] At the time of this Account, enzymological and crystallographic research, spearheaded by the group of Rosenzweig, has seemingly narrowed the focus to a dicopper active site in which one copper is coordinated by two histidine imidazoles and the other is chelated by an N-terminal histidine (Figure 1b).[41,45]

Motivation for the detailed study of this reaction, methane to methanol conversion at ambient temperature and pressure, is clear; however, precise information on the activation of dioxygen and hydroxylation by pMMO has not been forthcoming.[46] Aspects of the active site are also puzzling, in that the dicopper site does not resemble those of other well-characterized copper- and dioxygen-dependent enzymes.[1,42] The minimal, planar ligation in an asymmetric site—with the unusual primary amine motif—inspire speculation as to the nature of the oxygenated and reactive intermediates. If parallels can be drawn to the high-valent diiron(IV) diamond oxide core

operative in soluble methane monooxygenase (sMMO),[47] perhaps an analogous formally four-electron oxidized dicopper-(III) bis(μ-oxide) site may be relevant in pMMO. However, the Cu(III) state has never been definitely observed in biology. It is important to note that substantial homology, specifically at the conserved histidine residues of the proposed active site, exists between pMMO and bacterial and archaeal ammonia monooxygenase (AMO),[48] a significant source of chemical energy in marine and terrestrial microbial domains.

A synthetic study of the Cu-dioxygen chemistry relevant to this active site requires high analog fidelity, as the nature of supporting ligands on copper strictly dictates the ultimate stability of different dioxygen coordinations with respect to each other.[3] A model that faithfully recapitulates the specific elements of an active site may credibly evidence structures pertinent to the catalytic cycle. For pMMO, this means incorporation of imidazole and primary amine moieties in the ligand.

The importance of "core capture", discussed above, as a synthetic route to both known and unknown complexes has become abundantly clear to our research. For the first time in copper-dioxygen chemistry, we have been able to prepare and characterize a family of high-valent Cu(III) complexes stabilized by diamine ligands,[14] bearing primary amines, and histamine ligands,[15] bearing both imidazole and primary amine groups. Only by stoichiometric addition of exogenous ligands to a preformed dicopper(III) bis(μ-oxide) coordinated by TMPD at −125 °C in MeTHF are compounds unprecedented in the field accessed — the first high-fidelity models providing compelling evidence of the nature of dioxygen reactivity in pMMO (Figure 12).
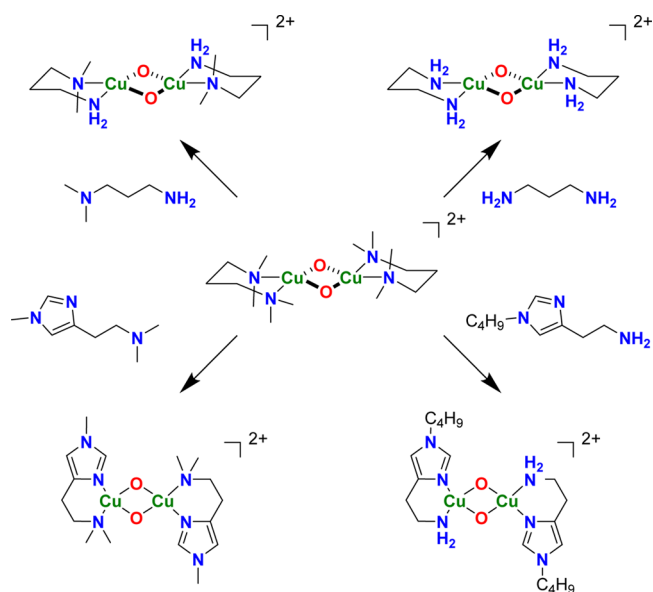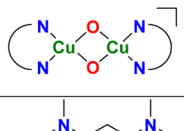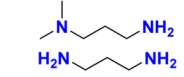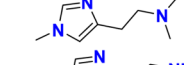

Figure 12. pMMO analogs through core capture.

Wholesale transfer of a high-valent Cu$_2$O$_2$ fragment represents a substantial structural rearrangement of an inherently reactive species. Proteolytic or oxidative susceptibility of imidazole or amino N−H or αC−H bonds on associating ligands opens irreversible decay pathways to the otherwise stabilized complex, and the ultimate longevity of the nascent complex, accessed by any route, was unknown. Productive formation and characterization of amino and

imidazole bonded Cu(III) compounds has simply never been observed in copper-dioxygen coordination chemistry.

Each of the core-exchanged dicopper(III) bis(μ-oxides) exhibits the two intense, characteristic LMCT optical bands (see Figure 2c) near 300 and 400 nm (Table 1). Each shows

Table 1. Metrics and Spectroscopic Properties of Bis(μ-oxide) Complex Family

| | UV-Vis λ, nm | Structural Metrics EXAFS[b] (DFT)[c] | | |
	(ε, mM^{-1} cm^{-1})[a]	Cu-Cu (Å)	Cu-O (Å)	Cu-N (Å)[d]
(tetramethyl diamine)	403 (29.4) 302 (18.7)	2.85 (2.83)	1.84 (1.81)	2.02 (2.00)
(dimethyl, NH$_2$)	390 (22.6) 290 (20.3)	2.82 (2.75)	1.85 (1.81)	2.01 (1.98 / 1.94)
(H$_2$N...NH$_2$)	375 (22.6) 278 (21.0)	2.77 (2.71)	1.86 (1.80)	2.00 (1.94)
(imidazole, NMe$_2$)	380 (30.2) 280 (24.5)	2.80 (2.77)	1.82 (1.81)	1.97 (1.98 / 1.91)
(imidazole, NH$_2$, C$_4$H$_9$)	363 (27.8) 262 (30.2)	2.78 (2.73)	1.82 (1.81)	1.96 (1.95 / 1.91)

[a]SbF$_6^-$, MeTHF, −125 °C, corrected ext. coeff. [b]Compounds **1–5** show Cu K pre-edge transitions at 8980.2, 8980.7, 8980.7, 8980.5, and 8980.6 eV, respectively, indicating a Cu(III) oxidation state. [c]Optimized at M06/TZVP/SMD(THF) level of theory. [d]DFT gives asymmetric Cu−N coordination for (Cu-NMe$_2$/Cu-NH$_2$) or (Cu−N$_{Amine}$/Cu−N$_{Imd}$).

clean pre-edge transitions near 8981 eV by Cu K edge XAS and diagnostically short Cu−Cu scattering interactions (~2.8 Å) by EXAFS.[8] DFT and TD-DFT calculations successfully reproduce all trends in spectroscopy and general metrical parameters.

The first notable spectroscopic trend among the complexes is the systematic blue-shifting of the optical bands with respect to ligation. For example, within the diamine series, removal of methyl substitution on nitrogen increases the energy of the "400 band" from 403 nm (tetramethyl) to 390 nm (dimethyl) to 375 nm (primary amine). In the histamine series, incorporation of imidazole blue shifts the spectra relative to the diamine series, and removal of amine methylation increases the absorption energy with respect to the TMPD complex (403 to 380 to 363 nm). Analogous blue-shifting is observed for the "300 band."

A simple mechanism of blue-shifting of the electronic excitations may implicate Cu−N contraction (Table 1). Primary amine and imidazole Cu−N ligation contracts with respect to Cu−N bonding by methylated ligands. This is easily interpretable through steric considerations. Analysis of the TD-DFT results indicates the energy of the characteristic LMCT transitions is tuned roughly by the energy of the accepting orbital in a donor-to-acceptor model of electronic transitions. Though the accepting orbital is rudimentarily metal-based, it exhibits significant Cu−N nitrogen antibonding character. Decrease of the Cu−N bond distance yields increased bonding

and consequently increased accepting orbital energy. In effect, the accessibility of the minimal amine and imidazole ligands toward copper make them *stronger* donors than a permethylated amine, contrary to approximations of metal−ligand bond strengths and orbital perturbations based on gas-phase proton affinity.[49]

The superior donation and increased metal−ligand bond strength are first identified by the optical spectra but are corroborated by the relative stability of each complex with respect to the others. Serial ligand exchange reactions reveal the thermodynamic stability rank of each complex (Figure 13). Within some experimental constraints, "single pot" exchanges can be conducted in which TMPD is displaced by exogenous chelating ligand, which is in turn exchanged by a second or third ligand. The generalized principle emerges that a primary amine is competent to displace a permethylated amine, and an imidazole is competent to displace both primary and tertiary amines. The resulting phenomenon is such that the most biological, least anticipated ligand with both imidazole and primary amine forms the most thermodynamically stable bis(μ-oxide) complex—all while maintaining the dicopper(III) state. Experimentally calibrated, DFT isodesmic ligand exchange reactions fully reproduce these thermodynamic trends.

An enhanced association of copper for imidazole over primary and tertiary amines is evident in crystallographic databases.[49,50] In almost all cases of Cu(II)-histamine or histidine complexes, Cu−N$_{imd}$ bond metrics are decreased with respect to those of Cu−N$_{amine}$. Indeed, this is identified almost as a generality among transition metals ligated by histidine or histamine ligands.

The systematic blue-shift of the bis(μ-oxide) spectra may bear some implication for previous investigation of oxygenated pMMO. Rosenzweig and co-workers observed a distinct absorption near 345 nm upon oxygenating truncated pMMO with either hydrogen peroxide or dioxygen.[51] The intensity was identified as problematic because it does not sufficiently differentiate between active dioxygen-bonded species[3] and an oxidized Cu(II) resting form. The energy is characteristic of a dicopper(II) μ−η^2:η^2-peroxide; however, the spectroscopy of our synthetic system unambiguously demonstrates encroachment of the dicopper(III) optical transition into dicopper(II) energy ranges. Definitive characterization of the enzyme active site will require more extensive spectroscopy or crystallography to identify the active intermediates in pMMO.

Minimization of the ligand steric demands in these complexes turns on reactivity. While no reaction rate is measured for the TMPD dicopper(III) bis(μ-oxide) with various exogenous C−H substrates at −125 °C, introduction of primary amine and imidazole groups allows the ligand exchanged complexes to oxidize C−H bonds (Table 2). The complexes show substrate-dependent, first-order decay in the presence of C−H(D) bearing substrates with substantial kinetic isotope effects, indicating rate-limiting C−H(D) bond cleavage.
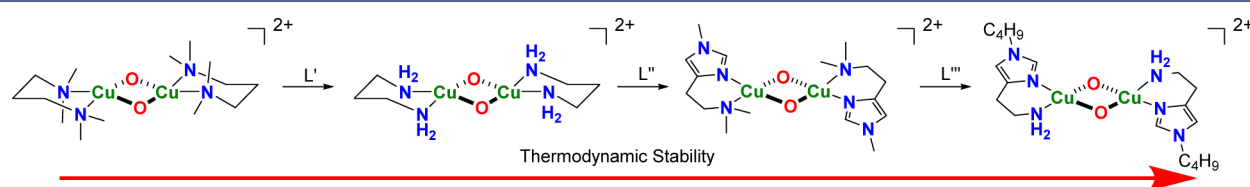

Figure 13. Rank-ordered stability of bis(μ-oxide) complex family.

Table 2. Substrate C−H(D) Oxidation Reactivity of Bis(μ-oxide) Family

	$BDFE \approx$ 74 kcal mol⁻¹	76 kcal mol⁻¹	76 kcal mol⁻¹
(TMPD)	No Rxn	No Rxn	No Rxn
	0.4 M⁻¹s⁻¹	--	--
	9.2 M⁻¹s⁻¹ KIE = 31	0.1 M⁻¹s⁻¹ KIE = 25	0.2 M⁻¹s⁻¹
	0.4 M⁻¹s⁻¹ KIE = 14	--	--
	4.6 M⁻¹s⁻¹ KIE = 20	0.3 M⁻¹s⁻¹ KIE = 31	0.1 M⁻¹s⁻¹

The rate of reaction is highly correlated with ligand sterics: the most accessible structures oxidize substrate with the greatest rate. Transition state optimization of C−H scission by a Cu_2O_2 core shows a linear association of the cleaved C−H bond with the O−O vector (Figure 14). This association pathway is highly hindered in the TMPD case, as no transition state can be found, nor reaction trajectory driven, computationally.
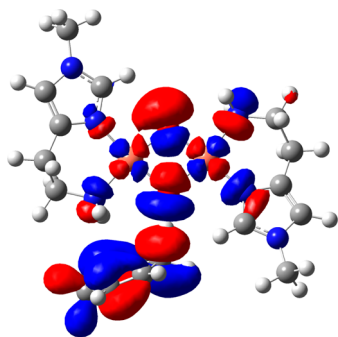
Figure 14. Transition-state orbitals of the histamine bis(μ-oxide) complex reacting with 1,4-cyclohexadiene.

The homolytic bond dissociation energies of the substrates explored here are weak.[52] However, a finite reaction rate is observed almost 150°C below room temperature. The reactivity investigation does not yet inform on the nature of the true oxidant, whether it is a bis(μ-oxide) or some subsequent, unidentified intermediate as the hydroxylating agent.[53] However, the conclusions are unambiguous: the dicopper(III) bis(μ-oxide) is distinctly stable and preferred within a proposed pMMO-like coordination sphere. The Cu(III) state is thermodynamically accessible from dioxygen, Cu(I), and histamine ligation.

The Cu(III) state has been excluded reasonably by others in mononuclear copper enzymes, as their reduction potentials most certainly fall outside of accepted biological windows.[54] However, the standard one-electron reduction potential of the histidine-based dicopper(III) bis(μ-oxide) may be accessible in our estimation. An extensive thermodynamic data set of polypeptide-derived, anionic amide-chelated Cu(III/II) couples has been assembled by Margerum,[55] providing an invaluable experimental calibration of in silico assessments of copper reduction potentials. Energy-calibrated DFT isodesmic reac-

tions, specific to copper in its +2 and +3 states, conservatively estimate the reduction potential of the histamine-bonded bis(μ-oxide) near 750 mV vs NHE, less than or equal to the outer-sphere reduction potential of the blue-copper site in fungal laccase.[56] Thus, exclusion of Cu(III)/Cu(II) states from biological relevance, on the basis of reduction potential, is potentially premature.[57,58]

Although the Cu(III/II) couple does not seem relevant to mononuclear copper in a protein environment, the unique attributes of the pMMO active site are conducive to the dicopper(III) bis(μ-oxide) oxygenated state. Planar disposition of a d^8-metal, absent of axial ligation, with strong donation by imidazole, primary amine, and anionic oxide ligands, and the exceptionally short Cu−Cu active-site separation (~2.6 Å),[59] make the dicopper(III) state the most credible first oxygenated intermediate in pMMO. Minimalism in the active site may kinetically facilitate the oxidation of methane. Intractabilities associated with investigation of purified pMMO continue to forestall definitive conclusions;[46,51] however, the distinct advantage of synthetic accessibility into relevant structures will hopefully narrow the breadth of the search.

6. CONCLUSIONS

Synthetic, small-molecule metal complexes and clusters are indispensable to the study of inorganic cofactors in biology. Often, the handles accessible by selective design and systematic perturbation provide the clarifying or revelatory information necessary to propose founded hypotheses. Experimentation with authentic protein can be difficult for many reasons. Synthetic models can fill the gap when established methodology does not yet exist for enzymological research. A triumph of bioinorganic chemistry was the first crystallographic assignment of the dicopper(II) $\mu-\eta^2:\eta^2$-peroxide[11] in a tris(pyrazolyl)borate coordination, some five years before the conclusive structural determination of the analogous oxy-hemocyanin active site,[13] prolifically modeled by chemists during the intervening years.[3] However, to many, lessons learned from genuine biological samples are definitive; synthetic systems provide proxy information credible only through the lens of analogy. The great theme emergent from chemical analysis of biology is that nature operates through and elaborates the reactivity permissible by the laws of chemistry. In bioinorganic chemistry, the ligation sphere is paramount. The judicious construction of a model may reveal what we do not know or reaffirm what is fundamental to function.

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Notes

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Sonja Herres-Pawlis, a native Westphalian, attended the Universities of Paderborn and Montpellier to obtain a diploma and Ph.D. in Chemistry. After her postdoctoral fellowship with T.D.P. Stack at

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T. Daniel P. Stack, a native Oregonian, attended Reed College (1977-1982) to obtain a B.A. in Chemistry and Harvard University (1982-1988) for his Ph.D. After his NSF postdoctoral fellowship at University of California, Berkeley, he joined the Stanford University Chemistry Department in 1991. His academic career has focused on synthetic analogues of biological metal sites, initially through the synthesis of elaborate ligands and more recently through the simplest of ligands at extremely low solution temperatures.

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