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Copper-Dioxygen Complex Mediated C-H Bond Oxygenation: Relevance for Particulate Methane Monooxygenase (pMMO)

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Summary

Particulate methane monooxygenase (pMMO), an integral membrane protein found in methanotrophic bacteria, catalyzes the oxidation of methane to methanol. Expression and greater activity of the enzyme in the presence of copper ion suggest that pMMO is a cuprous metalloenzyme. Recent advances – especially the first crystal structures of pMMO – have energized the field, but the nature of the active site(s) and the mechanism of methane oxidation remain poorly understood – yet hotly contested. Herein the authors briefly review the current understanding of the pMMO metal sites, and discuss advances in small molecule Cu-O₂ chemistry that may contribute to an understanding of copper-ion mediated hydrocarbon oxidation chemistry.

Introduction

Particulate methane monooxygenase (pMMO) is an integral membrane protein catalyzing the hydroxylation of methane, which possesses the strongest known aliphatic C-H bond (Bond Dissociation Energy = BDE = 104 kcal mol⁻¹). The enzyme is expressed by all (but one) species of methanotrophs. In species that also express the iron-containing soluble methane monooxygenase (sMMO) [1], sMMO expression occurs only in growth media with Cu concentrations < 0.8 μM; pMMO is expressed above a low (4 μM) threshold Cu concentration [2,3]. Copper is necessary for activity, and increased copper concentration up to 60-80 μM in the growth medium correlates with greater activity in cell-free preparations [4]. Currently, details of how copper would accomplish methane oxidation are largely unknown.

Copper-dioxygen C-H bond activation studies have intensified in recent years with interest stemming from pMMO-mediated oxidations and reactivity catalyzed by other copper oxygenases. While there is a considerable literature of copper-ion promoted organic oxidations [5,6], we here emphasize more recent efforts to describe copper-dioxygen or derived complexes and their reactivity. A hallmark of such studies is employing ligands that direct the ensuing Cu^I/O₂ structure and reactivity. This approach has produced a number of discrete Cu_xO_y complexes, which have been structurally and spectroscopically described (Figure 1) and surveyed for reactivity toward organic substrates [7,8]. One structure, the ‘cupryl’, has been suggested but not observed, though theoretical studies point to an unexpectedly high reactivity with respect to C-H bond oxidation [9-11].

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Which Cu-O species, if any, is responsible for methane oxidation? The location, structure, and nuclearity of the pMMO active site(s) remain contentious points. Small-molecule Cu-O reactivity studies have delineated the capabilities of specific Cu_xO_y structural types for C-H activation and may thus give clues to the identity of the active site. However, an understanding of the actual metal sites of pMMO must provide some context.

The pMMO Metal Sites

The first pMMO x-ray structure (2005, from *M. capsulatus* Bath) marked a breakthrough in the field [12]. The enzyme assembles as a heterotrimer of three subunits – pmoA, pmoB, and pmoC – with a solvent-exposed domain above the trans-membrane domain, which consists of α -helices (Figure 2). Prior studies had not predicted the trimeric structure, yet the shape and volume of membrane-bound enzymes, as determined by electron microscopy, support the biological relevance of the structure [13].

From there, certainty drops off precipitously. Three sites were identified as metal-containing: a two-coordinate (His_2) mononuclear copper site; a zinc site (likely adventitious, as the crystallization medium required the metal; purified pMMO does not contain zinc [14]) in the trans-membrane domain; and an anomalous site modeled as a dinuclear copper cluster (Figure 2). The cluster has one Cu ion bound by two His imidazoles; the second, by an imidazole and amino group of the pmoB N-terminal His. EXAFS analysis indicated a close Cu-Cu interaction in all redox states of the enzyme, thus supporting the dinuclear Cu-Cu assignment [15].

A more recent (2008) second crystal structure (pMMO from *M. trichosporium* OB3b) contained metal sites that differ from those described for *M. capsulatus* (Bath) [14]. The mononuclear copper site is absent (one His is not conserved), and the zinc replaced by a copper ion. The dinuclear center – with strictly conserved binding residues – is retained.

Are these metal sites biologically relevant [16]? Some researchers argue that the majority of pMMO's Cu ions are stripped during isolation/purification. Chan and coworkers propose the presence of at least one trinuclear copper cluster in cellular pMMO [17] on the basis of an isotropic EPR signal, along with redox-potentiometric titration and assorted other data [17, 18]. Computational modeling of a trinuclear cluster into the X-ray structure (Bath) is shown in Figure 2; the metal ions are bound by a set of hydrophilic residues (His, Glu; Met, Asp; Asp, Glu) clustered together in the trans-membrane domain (Figure 2) [18].

No other research groups have observed this cluster EPR signal, and alternative explanations for its existence have been suggested [16,19,20]. At least one Type 2 Cu center (i.e., 'normal' tetragonal Cu^{II} with imidazole ligands) is supported by all data, however. Another minority of researchers advocate the existence of a diiron site, similar to sMMO, based on Mössbauer [21] or EPR [22] spectroscopy. On the basis of the overall literature, however, the present authors believe pMMO possesses a copper-containing active site, and herein discuss the possibility of $\text{Cu}_x\text{-O}_y$ -promoted methane oxidation.

Modeling Studies of C-H Bond Activation

With the uncertainty surrounding the actual identity of the pMMO active-site structure, the oxidative capabilities of various small-molecule, O_2 -derived $\text{Cu}_x\text{-O}_y$ complexes may serve to evaluate the possibilities for an active species capable of hydroxylating methane. In addition to Chan's proposal for a trinuclear copper cluster, the dinuclear and mononuclear copper sites in the pMMO X-ray structures serve as candidates for the active site. In the following sections, we survey the known oxygen adducts for complexes possessing three, two, or one copper ion (s) and their known reactivity toward C-H bond-containing substrates.

TRINUCLEAR

Well-characterized trinuclear, O₂-derived copper-oxo species are rare [23,24]. Stack and co-workers reported the first [25], a system with two μ_3 -oxo ligands, formed via O-O bond cleavage of dioxygen by three Cu^I ions (Figure 3). The Cu ions are in the +2, +2, and +3 oxidation states, all bridged by two μ_3 -oxo ligands. Such a species was demonstrated by Itoh and co-workers to oxidize phenolic substrates [24]. Dihydroanthracene oxidation was reported as well. However, such complexes seem to possess limited reactivity.

A number of interesting trinuclear Cu clusters have been synthesized with the goal of rationally modeling the electronic properties of a putative trinuclear Cu cluster in pMMO [26]. In addition, there have been several reports of mild oxidative functionalization of alkanes – primarily cyclohexane (BDE ~ 97 kcal/mol) – by multinuclear Cu^{II}-complexes supported by triethanolamine (or other chelating) ligands, plus H₂O₂ [27,28]. Mechanistic details and the identities of active copper oxidants are poorly understood in these systems. Chan and co-workers, however, have reported an attempt at rational functional modeling of a putative trinuclear copper pMMO site (Figure 3) [17,29]. The Cu^I₃ complex – unstable toward oxidative decomposition – aerobically promotes conversion of benzil to its anhydride (identified by hydrolysis products). In the absence of substrate, the complex may oxidize acetonitrile to glycolonitrile (identified solely by ESI-MS and IR). The authors propose a singlet “oxene” insertion pathway (Figure 3) – in line with DFT calculations for their proposed pMMO methane hydroxylation mechanism [30] – but without supporting mechanistic studies.

DINUCLEAR

The trinuclear-cluster hypothesis remains controversial. The best evidence (highly-conserved residues, two X-ray structures, supporting EXAFS and EPR data), though inconclusive, supports a dinuclear copper cluster in pMMO. Activation of dioxygen by two copper ions leads to one of several structures. Our discussion here emphasizes the μ - η^2 : η^2 -peroxo-Cu^{II}₂ and the bis- μ -oxo-Cu^{III}₂ adducts (Figure 1). While isomeric they are spectroscopically distinctive and possess varying reactivity patterns [7,8,23,31]. A third structure, the end-on μ -1,2-peroxo-Cu^{II}₂ species (Figure 1), is nucleophilic and shows limited oxidative reactivity [7,32]. We now discuss μ - η^2 : η^2 -peroxo-Cu^{II}₂ and bis- μ -oxo-Cu^{III}₂ complexes in light of the pMMO dinuclear site, their oxidative reactivity toward C—H containing substrates and relevant theoretical studies.

The two structures, with a low barrier for interconversion, exist in an equilibrium (affected by several factors). The bis- μ -oxo-Cu^{III}₂ core possesses a Cu...Cu separation of ~ 2.8 Å, much shorter than that observed for μ - η^2 : η^2 -peroxo-Cu^{II}₂ complexes (~ 3.6 Å) [8,23]. Could then the pMMO dinuclear center, with comparably short Cu-Cu distance (~ 2.6 Å, by EXAFS [15]), in an activated state be capable of giving rise to a bis- μ -oxo-Cu^{III}₂ active-site capable of C—H oxidation chemistry? On the other hand, a bis- μ -oxo-Cu^{III}₂ structure has never been observed in enzymatic systems.

Numerous examples of μ - η^2 : η^2 -peroxo-Cu^{II}₂ and bis- μ -oxo-Cu^{III}₂ complexes that promote C—H bond oxidation have been reported. Most commonly, oxidation is a course for Cu₂O₂ thermal decomposition and occurs intramolecularly (i.e., via attack on a ligand bond) at carbon atoms alpha to a nitrogen atom or at benzylic C-H bonds. N-dealkylation occurs upon oxidation/hydroxylation of a α -NC-H bond; a subsequent transformation affords a 2° amine and aldehyde or ketone. Well-defined bi-, tri-, and tetradentate ligand-containing Cu₂O₂ systems have been shown to undergo oxidation at benzyl, isopropyl (methine), ethyl (methylene) and even methyl amine α -C-H bonds [8].

Kinetic and structural studies have elucidated the mechanistic details of bis- μ -oxo- Cu^{III}_2 core-promoted ligand N-dealkylation. Several factors come into play. Proximity of substrate C-H bond – based on ligand geometric constraints – for an in-plane approach to the oxo ligand determines the point of attack, as demonstrated by the example in Figure 4 [33,34]. Theoretical frontier molecular orbital studies revealed an oxygen-centered σ^* LUMO in the bis- μ -oxo- Cu^{III}_2 core plane, consistent with the selectivity of C-H attack [35]. Where different substituents have equal access to the core (as with a bis- μ -oxo- Cu^{III}_2 species supported by N,N-diaryl-diaminocyclohexane ligands, complexes which possess pseudo- C_2 symmetry) attack occurs at a C-H bond with the lowest BDE [36]. This observation pointed to an H-abstraction mechanism. In fact, large deuterium kinetic isotope effects (KIE) and expected activation parameters strongly support an H-abstraction pathway for bis- μ -oxo- Cu^{III}_2 -promoted N-dealkylation chemistry across a wide range of ligands [8].

Mechanistic studies of N-dealkylation in μ - η^2 : η^2 -peroxo- Cu^{II}_2 complexes gave entirely different activation parameters and, in fact, indicated that C-H abstraction is not the rate-determining step; rather, the peroxo complex must isomerize to the bis- μ -oxo- Cu^{III}_2 core in order to perform the reactivity [24]. The same was found to be true for intramolecular benzylic C-H hydroxylation chemistry. Two exemplary ligand systems for this reaction are shown in Figure 5. Ligand type L^{X} complexes form bis- μ -oxo- Cu^{III}_2 ; type $\text{L}^{\text{X'}}$, μ - η^2 : η^2 -peroxo- Cu^{II}_2 . For the μ - η^2 : η^2 -peroxo- Cu^{II}_2 complex, small KIE and relative insensitivity of the hydroxylation rate to para-substituents (X) on the phenyl ring were observed. The bis- μ -oxo- Cu^{III}_2 complex gave a high KIE and a Hammett correlation consistent with an H-atom abstraction pathway [37]. This evidence suggests that the μ - η^2 : η^2 -peroxo- Cu^{II}_2 species itself does not perform C-H abstractions; instead, rate-determining isomerization to an active bis- μ -oxo core is favored [38].

These results would suggest that methane hydroxylation at a copper dinuclear site, via C-H abstraction, would likely involve a bis- μ -oxo- Cu^{III}_2 rather than a μ - η^2 : η^2 -peroxo- Cu^{II}_2 intermediate. While μ - η^2 : η^2 -peroxo- Cu^{II}_2 complexes are capable of intramolecular arene hydroxylations, and even ortho-hydroxylation of exogenous phenolate substrates (which may bind to copper), these reactions occur via an electrophilic substitution pathway as opposed to formal arene C-H activation/abstraction. Consideration of the Frank-Condon barriers for C-H abstraction by the two structures, moreover, reveals a much higher barrier for μ - η^2 : η^2 -peroxo- Cu^{II}_2 than bis- μ -oxo- Cu^{III}_2 ; the transition state (T.S.) for the latter possesses much more reactant-like character [39].

Two aspects of the chemistry discussed above deserve scrutiny, however. First, the examples of intramolecular bis- μ -oxo- Cu^{III}_2 -promoted oxidations involve only activated C-H bonds. Second, close proximity of the oxidized C-H bond to the Cu_2O_2 core is required. Chan has emphasized the importance of tight active site-substrate organization in pMMO, with regard to its selectivity for substrate size (C_1 - C_5) in addition to the high regio- and stereoselectivity of its oxygenation chemistry [17,40]. However, N-dealkylation and benzylic hydroxylation reactions occur at C-H bonds much weaker than methane's and may have little significance for methane oxidation. Tellingly, intermolecular oxidation of hydrocarbon bonds by bis- μ -oxo- Cu^{III}_2 complexes is rarer and less understood. Some bis- μ -oxo- Cu^{III}_2 complexes and, in fact, some μ - η^2 : η^2 -peroxo- Cu^{II}_2 complexes – supported by ligands based on the bis(pyridylethyl) amine scaffold – are known to oxidize exogenous substrates such as N,N-dimethylaniline and THF, for example [41]. However, mechanistic studies suggest that these reactions are frequently initiated by heteroatom oxidation via electron transfer and/or facilitated by substrate binding to copper, pathways not available for methane hydroxylation. These complexes cannot oxidize purely hydrocarbon substrates, even those with weaker C-H bonds such as cyclohexadiene (BDE $\sim 77 \text{ kcal mol}^{-1}$) and dihydroanthracene (BDE $\sim 78 \text{ kcal mol}^{-1}$).

A bis- μ -oxo- Cu^{III}_2 species supported by a bidentate pyridylethylamine ligand, on the other hand, does oxidize cyclohexadiene and dihydroacridine via a C-H abstraction mechanism, as determined by KIE [42]. But a rate law with 2nd-order dependence on $[\text{Cu}_2(\text{O})_2]$ led the researchers to propose a high-valent (intuitively, more oxidizing) $\text{Cu}^{\text{IV}}(\text{O})_2\text{-Cu}^{\text{III}}$ active species, formed by pre-equilibrium $\text{Cu}_2(\text{O})_2$ disproportionation, for the reaction (Figure 6). Bis- μ -oxo- Cu^{III}_2 species are the proposed intermediates for catalytic (though low-yield) oxidations of the stronger C-H bonds of cyclohexane accomplished by copper complexes of β -diketeminate or bis(2-picolyamine) ligands [43,44]. However, these reactions utilize large excesses of H_2O_2 or $t\text{BuOOH}$ and the actual intermediates and kinetic pathways have not been determined.

Bis- μ -oxo- Cu^{III}_2 complexes supported by tripodal tetradentate ligands provide two of the few examples of exogenous hydrocarbon oxidation (toluene and ethylbenzene to benzaldehyde and acetophenone, respectively) promoted directly by bis- μ -oxo- Cu^{III}_2 species [45,46]. These four-electron reactions may proceed by complicated, multi-step pathways, but deuterium isotope effects are indicative of a rate-determining C-H cleavage event. The ground-state Cu_2O_2 structure of one of these complexes (Figure 7) is actually the end-on μ -1,2-peroxo- Cu^{II}_2 form; investigations suggested that this core must first isomerize to the bis- μ -oxo- Cu^{III}_2 structure in order to initiate benzylic C-H oxidation.

The latter two bis- μ -oxo- Cu^{III}_2 complexes are supported by picolylamine-type ligands rather than (pyridylethyl)amine ligands. Thus, generalizations about the reactivity of a specific Cu_2O_2 core without considering the effects of the ligand are inherently difficult. A μ - η^2 : η^2 -peroxo- Cu^{II}_2 complex supported by a dinucleating picolylamine-type ligand provides a uniquely encyclopedic example of these difficulties (Figure 8) [47]. The complex promotes intramolecular arene hydroxylation, similar to other μ - η^2 : η^2 -peroxo- Cu^{II}_2 complexes; the complex can oxidize C-H bonds (e.g., in THF) via a C-H abstraction mechanism, similar to bis- μ -oxo- Cu^{III}_2 complexes; and the complex provides the first (known) example of Cu_2O_2 -promoted alkene epoxidation. Small (< 5 carbons) olefins are epoxidized by pMMO as well, a fact suggesting that this μ - η^2 : η^2 -peroxo- Cu^{II}_2 species may have special significance for understanding pMMO chemistry. Specifically, arene hydroxylation and olefin epoxidation resemble (superficially, at least) the putative “singlet oxene” insertion mechanism proposed by Chan for pMMO (Figure 3) [30]. Activity studies of pMMO give a relatively low KIE (but still, at 5.2-5.4, suggestive of C-H cleavage in the rate-determining step) [17,48,49]; this result, along with the observed total retention of configuration during pMMO oxidation of chiral hydrocarbons, potentially points to a non-radical pMMO mechanism such as O-atom insertion into the methane C-H bond. In the case of the complex in Figure 8, however, an extraordinarily high KIE of 50 was found. In addition, oxidation of stronger C-H bonds by this complex has yet to be reported; further studies are necessary to evaluate its significance for understanding pMMO.

A question may be raised at this point: Are there any examples of Cu_2O_2 -promoted oxidation of stronger, unactivated C-H bonds? There are, in fact, for several intramolecular reactions involving oxidation of an $i\text{Bu}$ methine C-H [50], an N-propylamine β C-H [51], and an $i\text{Pr}$ methyl C-H bond [52]. Kinetics supported an H-abstraction mechanism in the first case (Figure 9, A) [50]. However, the other two examples provide hints concerning the potential significance of a mixed-valent $\text{Cu}^{\text{II}}(\text{O})_2\text{-Cu}^{\text{III}}$ species – suggested in calculations by both Yoshizawa and Chan to be more reactive toward C-H bonds than bis- μ -oxo- Cu^{III}_2 [30,49]. Propyl oxidation in an $n\text{Pr}$ -PY2 ligand system (Figure 9, B) occurs only in the presence of excess benzoin reductant and only after long reaction times at room temperature, precluding detailed investigation of the Cu_2O_2 intermediate(s). However, the need for a reductant led the authors to propose a mixed-valent $\text{Cu}^{\text{II}}\text{-Cu}^{\text{III}}$ active species formed by electron transfer to the initial Cu_2O_2 complex [51]. A similar species was invoked to explain the methyl C-H oxidation

observed in Figure 9, C [52]. Dioxygen and phenol are required; otherwise, no ligand oxidation occurs. The authors proposed that a fleeting mixed-valent $\text{Cu}^{\text{II}}(\text{O})_2\text{-Cu}^{\text{III}}$ species performs C-H abstraction, affording a carbon-centered methyl radical that rapidly reacts with O_2 . While supporting evidence for the proposal has not been forthcoming, it is in accord with the DFT studies (mentioned above) suggesting that injection of an electron into the bis- μ -oxo- Cu^{III}_2 core significantly increases its oxidative reactivity [9,53]. Computational studies by Chen and Chan corroborate this increased reactivity, though they reject it in favor of a $\text{Cu}_3\text{-O}_2$ active species due to KIE and selectivity considerations; the $\text{Cu}^{\text{II}}(\text{O})_2\text{-Cu}^{\text{III}}$ species, a spin-doublet system with a high-energy singly-occupied HOMO, would likely go through a C-H abstraction/radical pathway [30].

Several points may now be raised in the context of the enzyme:

-- Bis- μ -oxo- Cu^{III}_2 complexes oxidize C-H bonds that approach the $\text{Cu}_2(\text{O})_2$ core with ease, either due to intramolecular proximity (i.e., a ligand bond or coordinating solvent/substrate) or high concentration (e.g., solvent). A cavity lined with hydrophobic residues (conserved) lies adjacent to the dinuclear site in pMMO, with another such cavity stretching into the trans-membrane domain [3]. The cavity may provide a hydrophobic binding pocket for methane, but it is currently unknown if these cavities can account for the specific approach of hydrocarbon substrate that leads not only to reactivity with the inert, non-binding substrate, but also for observed selectivity (in terms of stereo- and regioselectivity, as well as substrate size). Chan and co-workers located a hydrophobic pocket near the “D” site (Figure 2); they propose that the dimensions of the site could account for the observed selectivities of the enzyme [18]. Perhaps an active di- (or tri-) nuclear site does in fact reside in close proximity?

-- Bis- μ -oxo- Cu^{III}_2 complexes oxidize C-H bonds significantly weaker than methane's, in accord with computational studies that locate a prohibitive barrier for bis- μ -oxo- Cu^{III}_2 -promoted methane hydroxylation [30,53]. A $\text{Cu}^{\text{II}}(\text{O})_2\text{-Cu}^{\text{III}}$ species (i.e., one-electron reduced bis- μ -oxo- Cu^{III}_2) would possess significantly greater reactivity according to these DFT studies. Further, a similar species – $\text{Cu}^{\text{II}}(\text{O})(\text{OH})\text{-Cu}^{\text{III}}$ – is a necessary intermediate of oxidation/abstraction reactions already known to be promoted by a bis- μ -oxo- Cu^{III}_2 moiety; the fact that it has never been observed (and immediately reacts upon formation) suggests that the species is “hot.” In pMMO, formation of such a species from copper(I) and dioxygen requires an additional electron, either from an exogenous reducing agent or, for example, from the protein itself. Another metal ion – either the third copper ion of a trinuclear center, or a copper ion in a distal (the mononuclear copper?) site – could be a source of electrons [12,54]. Or a tyrosine residue could provide an electron. In fact, Tyr374 resides ~5 Å from the dinuclear Cu site in the Bath enzyme [12]. However, this residue is not conserved, and no other Tyr is within 10 Å of the site in the OB3b structure [14].

While theoretical studies have determined methane H-abstraction transition states involving $\text{Cu}^{\text{II}}(\text{O})_2\text{-Cu}^{\text{III}}$, another possibility is cleavage of the dicopper-oxo core upon reduction to expose a terminal cupryl species (i.e. $\{\text{Cu}^{\text{II}}(\text{O})(\text{OH})\text{-Cu}^{\text{III}}\} \rightarrow \{\text{Cu}^{\text{II}}\text{-OH}\dots\text{Cu}^{\text{II}}\text{-O}\bullet\}$), also suggested by DFT calculations to be reactive toward methane. This and other mononuclear species are the focus of the final section of this survey.

MONONUCLEAR

The possibility of methane hydroxylation at a mononuclear copper site (or at one Cu ion of a dinuclear center) is the focus of our final considerations. A number of Cu-O_2 species have been crystallographically and/or spectroscopically characterized (Figure 1) – end-on $\text{Cu}^{\text{II}}\text{-O}_2^-$, side-on $\text{Cu}^{\text{II}}(\eta^2\text{-O}_2^-)$, and side-on $\text{Cu}^{\text{III}}(\eta^2\text{-O}_2^{2-})$. These species, however, possess limited oxidative reactivity (e.g., just oxidation of phenols) [8,55-57]. Several mononuclear Cu-

hydroperoxo species are known, but DFT studies suggest that this moiety, as well as a $\text{Cu}^{\text{II}}(\text{O}_2^{\bullet-})$ species, are incapable of oxidizing methane [9-11].

Theoretical studies do suggest, however, that a mononuclear copper-oxo (“cupryl”, Figure 1) may possess the ability to oxidize methane [9,10]. The species has not been observed in any molecular or enzymatic system, but indirect evidence has led several researchers to suggest such a species as a reactive intermediate. Several instances – experimental and theoretical – of intramolecular arene hydroxylation have been proposed to be promoted by a cupryl [58-60]. For example, oxygenation of a mononuclear $\text{Cu}(\text{I})$ - α -ketocarboxylate complex leads to intramolecular hydroxylation of a pyridyl phenyl arm (Figure 10). A computed pathway involves O-O bond cleavage in the peracid to afford the cupryl, which then electrophilically hydroxylates the ligand phenyl substituent. [Another pathway is attack by the peracid itself on the phenyl ring, though the T.S. maintains significant “copper-oxo character.”]

More significantly for pMMO, aliphatic C-H bond activation – intra- and intermolecular – has been proposed for cupryl intermediates. A Cu^{II} -cumylperoxo complex (Figure 10) oxidizes cyclohexadiene to benzene (without ligand hydroxylation), with O-O bond cleavage again (this time, from the alkylperoxo moiety) affording a cupryl as the proposed active oxidant. The observation of acetophenone (from β -scission of cumyloxy radical) and a “radical-trapped” Cu-DMPO alkoxyl adduct provided some evidence for the suggested homolytic cleavage pathway [61]. A cupryl entity has been suggested/implicated in oxidations initiated by Cu^{II} -OOH species, as well. Electron transfer or O-O homolysis would afford the cupryl [55,62]. In one case, the reactivity results in intramolecular hydroxylation of a ligand methyl (α to nitrogen) C-H bond (Figure 11, lower right) [55].

However, the implications of the several pathways (Figure 11) to oxygenated product via a “hot” cupryl pose problems in the context of the enzyme. The “concerted” route may be considered alongside a pathway involving initial H-abstraction by Cu^{II} -OOH to give water and cupryl, which then rebounds with the ligand carbon-centered radical to give product. It has already been mentioned above that the Cu^{II} -OOH moiety is not capable of performing this reactivity with methane as substrate. Initial homolytic cleavage of Cu^{II} -OOH entity would give the cupryl and hydroxyl radical. The regio- and stereoselectivity of pMMO hydrocarbon oxidation argue against formation of a diffusible, highly reactive free radical, however. (In the Itoh cumylperoxo system – Figure 10 – the methyl radical that must form following cumyloxy radical decomposition is unaccounted for.) Additionally, theoretical thermodynamic considerations suggest that this route is not feasible due to the unfavorable energetics for O-O homolysis [11]. Heterolytic cleavage of Cu^{II} -OOH would give hydroxide (avoiding the problem of diffusible radical formation) and a super high-valent $\text{Cu}^{\text{IV}}=\text{O}$ moiety, which could directly insert into the C-H bond. Yet such a species is without any precedent or theoretical support.

These observations highlight difficulties for rationalizing a cupryl species at a solely mononuclear copper site as the pMMO active species. In addition, a single copper(I) ion cannot provide the necessary three electrons to directly form a cupryl from O_2 . Alternative sources of electrons, to reduce an initial copper(II)-superoxide ($\text{Cu}^{\text{II}}\text{-O}_2^{\bullet-}$, i.e., a copper(I)-dioxygen adduct) to the cupryl and water, are possible. Yoshizawa has hypothesized that sequential reduction of $\text{Cu}^{\text{II}}\text{-O}_2^{\bullet-}$ by two Tyr residues in pMMO might form a cupryl at the mononuclear site [9]. A similar proposal for cupryl formation at the Cu_M site in dopamine β -monooxygenase was subsequently discarded based on mutagenesis studies [63]. In addition, while Tyr50 or Tyr330 are close to the Bath mononuclear site, the “zinc” site (the more likely location of a mononuclear Cu ion) is 10 Å from the two closest Tyr residues (although two Tyr residues, at distances 10-12 Å, are strictly conserved across pMMOs – see sequences in the Supporting Information to [2]). Another possibility is that the dinuclear site provides reducing equivalents.

Rosenzweig and co-workers have noted that the pMMO dinuclear Cu_2 site and the Cu_A site (electron transfer site) of cytochrome *c* oxidase reside in subunits of similar structure and are positioned similarly above the trans-membrane domains of their respective subunits) [12]. Yet the ligand complement of the pMMO dinuclear site is unlike any known for enzymatic copper electron-transfer sites; and a two-electron transfer, by means unknown, would be necessary to reduce an initial $\text{Cu}^{\text{II}}\text{-O}_2^-$ to a cupryl. Therefore, given the “tools” currently known to be available to pMMO, the formation of cupryl at an isolated mononuclear site seems unlikely, making a di- or trinuclear copper active site attractive as the actual active site.

Conclusions and Outlook

Based on the current literature (surveyed above), the authors believe/propose that a multicopper-dioxygen active site (Figure 12) is the best candidate for the methane hydroxylation catalyst in pMMO. From there, several hypotheses and proposals for future research may be broached on the basis of what is now known about copper-dioxygen complexes and pMMO itself. These include:

- Theoretical and experimental hints (Figure 9) suggest that the oxidation of very strong C-H bonds by bis- μ -oxo- Cu^{III}_2 first requires an additional electron. A reasonably characterized dioxygen-derived, mixed-valent ($\text{Cu}^{\text{II}}\text{-Cu}^{\text{III}}$ or $\text{Cu}^{\text{III}}\text{-Cu}^{\text{IV}}$) bis-oxo (or oxo-hydroxo) complex has yet to be observed. Such a species would be an important synthetic target for modeling studies.
- Computational studies also point to exceptional oxidative reactivity for a cupryl, possibly making it capable of oxidizing methane. Again, such a species may be relevant to pMMO if a mononuclear site is catalytic. Alternatively, a cupryl species (i.e., terminal Cu-oxo) may be generated at one copper ion within a multinuclear cluster (Figure 12) [51]. Further investigation of the nature of a cupryl species is required.
- The pMMO trinuclear cluster remains controversial. Its complete absence from the enzyme is far from unlikely, yet given the scarcity of O_2 -derived trinuclear Cu-O model complexes, it is a hypothesis that deserves more attention in the bioinorganic field. Additionally, Chan and co-workers propose an unprecedented set of amino acid ligands for the trinuclear cluster, with several carboxylates in addition to methionine and histidine. The mononuclear Cu ion discovered in the OB3b structure may also be bound by carboxylates in addition to His residues. Nitrogenous donor ligands have dominated bioinorganic modeling of enzymatic copper ions. The investigation of new ligand sets may be required before more is understood about copper-promoted hydrocarbon oxidation.

The last point raises the possibility that, despite the extensive reactivity of known Cu-O species toward internal and exogenous substrates, understanding *relevant* Cu-O structure and reactivity may require entirely new directions of research in copper-dioxygen chemistry. For the moment, though, little can be said definitively until studies of the enzyme itself reveal more about the metal sites and their oxygenated structures. Elucidation of methane hydroxylation by copper will likely be a collaborative effort between the enzymology and chemistry fields, and promises to be lively for years to come.

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Figure 1. Crystallographically or spectroscopically characterized Cu-O₂ adduct structures found in small-molecule ligand-copper complexes, with characteristic reactivity patterns (**green**). The "cupryl" species has been hypothesized and treated computationally, but has not been isolated for direct study.

Figure 2.

Far left: Ribbon diagram of the X-ray structure of a single protomer (pmoA, pmoB, and pmoC) in pMMO from *M. capsulatis* (Bath). Center left: The metal sites found in this X-ray structure, including a) a dinuclear copper cluster (B site), b) a mononuclear copper center (A site), and c) a zinc ion. Center right: Relative positions and distances between the metal sites and a “D” site cluster of hydrophilic residues. Right: A trinuclear $\mu_3\text{-O-Cu}^{\text{II}}_3$ cluster computationally modeled into the “D” site. Adapted from Refs. 16,18.

Figure 3.

Top: The cationic portion of the crystal structure of a $\mu_3\text{-O-Cu}_3$ complex, formed by the four-electron reduction of dioxygen by three copper(I) complexes possessing nitrogenous ligands. Middle: The cationic portion of a model complex for the proposed (yet controversial) pMMO trinuclear copper cluster model. Bottom: Diagram of the “oxene insertion” mechanism for methane hydroxylation proposed by Chen and Chan. Arrows represent electron spins. Adapted from Refs. 23,29.

Figure 4.

Oxidative N-dealkylation chemistry promoted by a representative bis-μ-oxo-Cu^{III}₂ complex, supported by a triazacyclononane (TACN) ligand. Bottom: Ball-and-stick model of the bis-μ-oxo-Cu^{III}₂ C-H abstraction transition state leading to N-dealkylation of a TACN benzyl group, as determined by DFT calculations. Adapted from Refs. 8,34.

Figure 5.

Intramolecular ligand hydroxylation at an ethylbenzene benzylic position, promoted by a bis- μ -oxo- Cu^{III}_2 complex (with ligand L^{X}) or a μ - η^2 : η^2 -peroxo- Cu^{II}_2 complex (with ligand $\text{L}^{\text{X}'}$). The bottom two chemical equations demonstrate the pathways for hydroxylation, showing that the (μ - η^2 : η^2 -peroxo- Cu^{II}_2) goes through rate-determining isomerization to a bis-oxo core before ligand oxygenation occurs.

Figure 6.

Disproportionation of the bis- μ -oxo- Cu^{III}_2 proposed by Fukuzumi and Itoh to rationalize a 2nd-order rate dependence on $[\text{Cu}_2(\text{O})_2]$ in the oxidation of exogenous C-H substrates.

Figure 7.

Exogenous toluene oxidation effected by a Cu₂O₂ complex supported by a tetradentate N₃O-donor ligand. Reaction of O₂ with the parent Cu(I) complex affords an end-on μ-1,2-peroxo-Cu^{II}₂ structure (left). Isomerization affords a bis-μ-oxo-Cu^{III}₂ (right) that is capable of oxidizing the toluene solvent benzylic C-H bonds.

Figure 8. Reactions promoted by $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo-Cu}^{\text{II}}_2$ complex supported by a xylyl-based dinucleating ligand. These reactions include arene hydroxylation – common for this class of compound – but also C-H bond activation of THF (generally thought to occur exclusively from a bis- $\mu\text{-oxo-Cu}^{\text{III}}_2$ species) and styrene epoxidation. This chemistry represents the first example of olefin epoxidation promoted by a Cu_2O_2 complex.

Figure 9.

Oxidation of unactivated aliphatic C-H bonds by several bis-μ-oxo-Cu^{II}₂ complexes. A: Oxidation of a methine C-H by a triaminocyclohexane-supported bis-μ-oxo-Cu^{II}₂ complex. B: Intramolecular methyl C-H oxidation, initiated by the reaction of phenol with a μ-η²:η²-peroxo-Cu^{II}₂ in an aerobic environment. C: Pathways to β C-H bond activation by a Cu^{II}-Cu^{III} bis-μ-oxo species proposed to form via benzoin reduction of the parent bis-μ-oxo-Cu^{II}₂ complex.

Figure 10.

Top: Computationally-determined pathways to arene hydroxylation following the reaction of O_2 with an α -ketocarboxylate-Cu^I complex. A copper-oxo species is proposed to perform an electrophilic substitution of the phenyl ring. Bottom: Proposed pathway to formation of an active cupryl complex via homolytic cleavage of an O-O bond in the parent Cu^{II}-cumylperoxo complex. Formation of a Cu^{II}-alkoxy complex by reaction of the parent with radical-trap DMPO was observed and cited as evidence for the existence and formation of the cupryl species.

Figure 11.

Formation of a Cu^{II}-OOH entity via hydrogen atom transfer to a Cu^{II}-O₂^{•-} complex, with possible pathways to the subsequent N-CH₃ hydroxylation reactivity. Homolytic cleavage of the O-O bond in Cu^{II}-OOH affords cupryl along with a hydroxyl radical. Heterolytic cleavage would give Cu^{IV}=O and hydroxide, with the latter effecting C-H bond insertion?

Figure 12.

The authors' most favored copper-O₂ derived entities which would effect strong C-H bond 'oxygen activation', i.e., R-H → R-OH. The electron source is likely to be a copper(I) ion, neighboring or more distant.