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Controlled Oxidation of Hydrocarbons by the Membrane-Bound Methane Monooxygenase: The Case for a Tricopper Cluster

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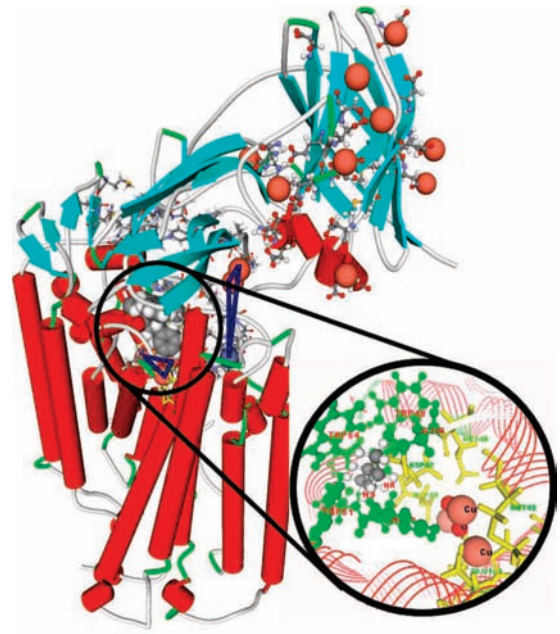
CONSPECTUS

The growing need for inexpensive methods to convert methane to methanol has sparked considerable interest in methods that catalyze this process. The integral membrane protein particulate methane monooxygenase (pMMO) mediates the facile conversion of methane to methanol in methanotrophic bacteria. Most evidence indicates that pMMO is a multicopper enzyme, and these copper ions support redox, dioxygen, and oxo-transfer chemistry. However, the exact identity of the copper species that mediates the oxo-transfer chemistry remains an area of intense debate. This highly complex enzyme is notoriously difficult to purify because of its instability outside the lipid bilayer and tendency to lose its essential metal cofactors. For this reason, pMMO has resisted both initial identification and subsequent isolation and purification for biochemical and biophysical characterization.



In this *Account*, we describe evidence that pMMO is a multicopper protein. Its unique trinuclear copper cluster mediates dioxygen chemistry and O-atom transfer during alkane hydroxylation. Although a recent crystal structure did not show this tricopper cluster, we provide compelling evidence for such a cluster through redox potentiometry and EPR experiments on the "holo" enzyme in pMMO-enriched membranes. We also identify a site in the structure of pMMO that could accommodate this cluster. A hydrophobic pocket capable of harboring pentane, the enzyme's largest known substrate, lies adjacent to this site.

In addition, we have designed and synthesized model tricopper clusters to provide further chemical evidence that a tricopper cluster mediates the enzyme's oxo-transfer chemistry. These biomimetic models exhibit similar spectroscopic properties and chemical reactivity to the putative tricopper cluster in pMMO. Based on computational analysis using density functional theory (DFT), triangular tricopper clusters are capable of harnessing a "singlet oxene" upon activation by dioxygen. An oxygen atom is then inserted via a concerted process into the C−H bond of an alkane in the transition state during hydroxylation. The turnover frequency and kinetic isotope effect predicted by DFT show excellent agreement with experimental data.

Introduction

The particulate methane monooxygenase (pMMO) is an integral membrane protein that mediates the facile conversion of methane to methanol in methanotrophic bacteria.[1] It also catalyzes the hydroxylation and epoxidation of a small number of straight-chain alkanes and alkenes up to

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five carbons in length.[2] Unlike the soluble methane monooxygenase (sMMO),[3–7] the chemistry mediated by pMMO exhibits amazing regiospecificity and stereoselectivity.[2,8–10] Both the hydroxylation and epoxidation occur with total retention of configuration at the carbon center(s) oxidized.[8–10] Accordingly, there is considerable interest in elucidating the structure of the active site of this enzyme.

There is now general consensus that pMMO is a multicopper enzyme,[1,11–18] although a nonheme diron center has been implicated recently as well.[19] These copper ions support redox, dioxygen, and oxo-transfer chemistry; thus there is no question that they are involved in the catalytic cycle of the enzyme. The exact identity of the copper species that mediates the oxo-transfer chemistry, however, remains an area of intense debate.[1,16,20,21] In this Account, we summarize the current status of our understanding of the structure and mechanism of the catalytic site. The discussion will focus on the pMMO isolated from *Methylococcus capsulatus* (Bath). A number of reviews have recently appeared in *Accounts of Chemical Research*[18] and elsewhere[22] on pMMO. However, these discussions have been based largely on a crystal structure of the protein with many of the copper cofactors missing.[16]

Subunit Composition and Metal Cofactors

pMMO is comprised of three distinct subunits encoded by the genes *pmoA*, *pmoB*, and *pmoC* with an $\alpha\beta\gamma$ arrangement.[11–15] The protein has been highly produced in *M. capsulatus* (Bath) and solubilized in the detergent dodecyl-β-maltoside from pMMO-enriched (80−90%) membranes, and the protein−detergent complex has been purified by size-exclusion chromatography.[1,12] The purified micelle contains one copy each of the three subunits encapsulated in ca. 240 detergent molecules. MALDI-TOF mass spectrometry has yielded three peptides with molecular masses of 42 785.52, 29 733.31, and 28 328.25 Da in excellent agreement with the subunit masses predicted by the gene sequences *pmoB* (α), *pmoC* (γ), and *pmoA* (β), respectively. The assignment has been confirmed by peptide-mass fingerprinting of in-gel digests of the subunits on SDS gels. Since the purified protein−detergent complex exhibits high specific activity toward propene epoxidation using either NADH or duroquinol to provide the reducing equivalents, it is clear that the *holo* enzyme has been purified in its active form.[12]

Analysis of the purified protein−detergent micelles for metal content using atomic absorption, inductively coupled plasma mass spectrometry (ICP-MS), and X-ray absorption edge spectroscopy revealed 13.6 copper atoms per protein complex (MW 100 kDa).[12] Only traces of iron were found (Cu/Fe atom ratio 80:1), and there was no evidence of any Zn.[12] A recent Mössbauer study has implicated a nonheme diiron center in whole cells of *M. capsulatus* (Bath), and suggested that the purified pMMO might contain this diiron center with 10% occupancy.[19] This level of Fe corresponds to the Cu/Fe content determined for the purified protein−detergent micelles reported from the Chan laboratory, for which the copper ions have been shown to support redox, dioxygen, and oxo-transfer chemistry. The turnover of the copper ions depends on the presence of hydrocarbon substrate, so a subset of the coppers must be involved in the oxidation of the substrate.

Our strategy to start with membranes highly enriched in the enzyme, solubilize the pMMO-enriched membranes in detergent, and fractionate the detergent−protein micelle particles by size-exclusion chromatography was critical to our success in obtaining high-quality protein for in-depth study.[12] Compared with conventional methods of membrane-protein isolation and purification by detergent solubilization, ammonium sulfate fractionation, or affinity chromatography,[13–15,23] our approach is simple and essentially nondisruptive of the protein fold and hence less prone to the loss of metal cofactors. It is now apparent that ammonium sulfate precipitation can leech out many of the copper ions from the pMMO. Although the addition of methanobactin[24] can replenish these copper cofactors, this copper-binding compound is not an integral component of the pMMO system. The pMMO with the full complement of copper ions does not require methanobactin for activity.

The Biochemistry of Substrate Oxidation by pMMO

pMMO hydroxylates straight-chain alkanes from C1 to C5 and epoxidizes related alkenes. In the case of propane, butane, and pentane, the dominant products are propan-2-ol, butan-2-ol, and pentan-2-ol, respectively.[2] As expected, 1-butene is hydroxylated to give 3-buten-2-ol and epoxidized to 1-epoxybutane, with a product distribution of ~1:1.[2]

Studies on cryptically chiral ethanes have shown that the hydroxylation of C−H and C−D bonds proceeds with total retention of configuration at the carbon center oxidized (Figure 1) with a k_H/k_D kinetic isotope effect of 5.2−5.5.[8] Similarly, experiments on butane and *d,l*-[2−$^2H_1$,3-$^2H_1$]butanes have shown that the hydroxylation of the secondary carbon in normal butane also proceeds with total retention of configuration, with a similar k_H/k_D isotope effect.[9] These observations have led us to rule out a radical mechanism for the

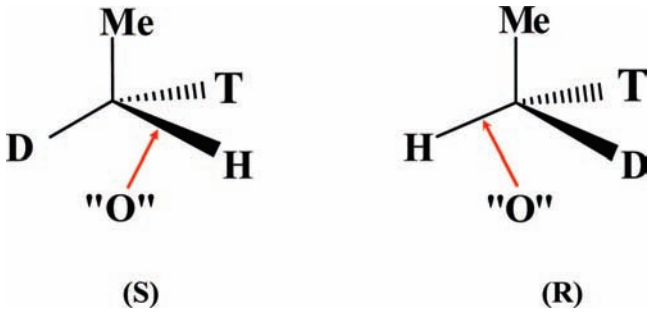


FIGURE 1. "Oxenoid" insertion into the C−H bonds of cryptically chiral ethanes.

hydroxylation chemistry. Instead, we have proposed that the O-atom transfer occurs by concerted oxenoid insertion.[8] A transition state involving "singlet oxene" insertion into a C−H bond should result in facile bond closure of the C−O bond following formation of the O−H bond, and the process should proceed with full retention of configuration at the carbon center oxidized.[1]

When propene and 1-butene were used as epoxidation substrates, the enantiomeric excess (ee) of the enzymatic products was only 18% and 37%, respectively.[2] However, studies on *trans*-2-butene revealed only the *d*,*l*-2,3-dimethyloxirane products and on *cis*-2-butene, only the *meso* product.[10] These latter observations indicate that the enzymatic epoxidation also proceeds via the concerted electrophilic *syn* addition. Thus, the relatively poor stereoselectivity in the enzymatic epoxidation of propene and 1-butene merely reflects the low stereochemical differentiation between the *re* and *si* face in the hydrophobic pocket of the active site. To achieve better facial selectivity, Yu et al. have recently used 3,3,3-trifluoropropene as the substrate.[10] The products obtained are 90% the *S*-oxirane.

The activity of pMMO is usually determined by the propene epoxidation assay. For pMMO-enriched membranes, membrane fragments solubilized in dodecyl-β-maltoside, and the purified protein−detergent complex in micelles, the specific activity is typically 10−100 nmol/(min·mg of protein).[1] It has been suggested that this level of activity of the purified enzyme is only ca. 10% of the activity in whole cells, where the protein would contain the diiron center implicated in the recent Mössbauer study.[19] Direct comparison between whole cell activity and activity assays of isolated membranes or purified protein in detergent particles in buffer is complex. First, in whole cells, the function of the enzyme is linked to an intricate electron transport system. In isolated preparations, the reductant(s) and the electron transfer pathways used in driving the pMMO activity may not be the physiological ones. In the recent Mössbauer study, the whole cell activities were assayed using formate as the reductant, and the purified enzyme was assayed using duroquinol. Formate is water-soluble, whereas duroquinol is membrane-bound. Two substrates are involved in pMMO. The metal cofactor(s) at the active site need to be activated by dioxygen, and the hydrocarbon substrate needs to get to the catalytic site of the pMMO. In whole cells, the protein is embedded in the membrane, and the substrates are distributed in different cellular locations within the cell. This distribution is different in suspensions of isolated membranes and solutions of the purified protein. Finally, it is difficult to control abortive or nonproductive processes outside the cellular environment. This control is obviously fine-tuned to perfection within the cell.

Proposed Arrangement of the Copper Ions and Their Role in the Catalytic Cycle

Because 6 of the 14−15 coppers in pMMO are oxidized when the reduced enzyme is reacted with dioxygen in the absence of hydrocarbon substrate, we have previously divided the copper ions into two groups:[25] a collection of six copper ions that are readily oxidized by dioxygen in the absence of hydrocarbon substrate and a second grouping of ca. nine copper ions that remain reduced under these conditions. The first group has been referred to as catalytic clusters (C-clusters) to reflect their reactivity toward molecular oxygen.[25] These copper ions are implicated in the dioxygen chemistry and the alkane hydroxylation of the enzyme. The second group of nine copper ions have been proposed to be sequestered into a Cu(I) domain to provide a reservoir of reducing equivalents to rereduce the copper ions at the active site after the oxidative phase of the turnover. This buffer of Cu(I) ions has been dubbed E-clusters.[25] These copper ions reside in the C-terminal subdomain of PmoB. Yu et al. have recently cloned and overexpressed both the N-terminal and C-terminal subdomains of the PmoB subunit in *Escherichia coli* and have shown that the purified C-terminal subdomain behaves like a Cu^{I} sponge, capable of binding 10 or more Cu^{I} ions cooperatively.[26] Because the C-terminal subdomain exhibits only weak binding for Cu^{II}, the redox potentials of these copper ions are high. These Cu^{I} ions are also inert toward molecular oxygen. Accordingly, they are normally EPR silent. These properties are identical to those noted for the E-clusters in the intact protein. Upon oxidation of the E-clusters, some of these copper ions are easily removed during purification and manipulation of the protein, with concomitant loss of activity.

It was originally proposed that the six copper ions associated with the C-clusters were organized into two trinuclear copper clusters.[1,25,27] A working model was developed for the

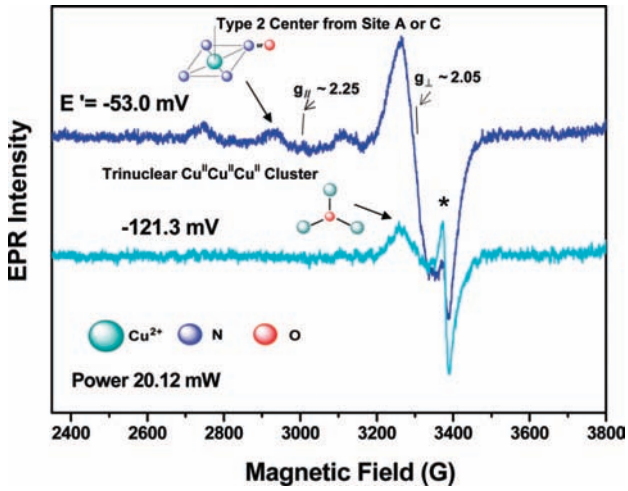


FIGURE 2. Redox potentiometry and EPR titration of the C-cluster copper ions. Asterisks in the spectra at $g \approx 2.002$ denote signals originating from free radicals associated with the dithionite and the redox mediators.

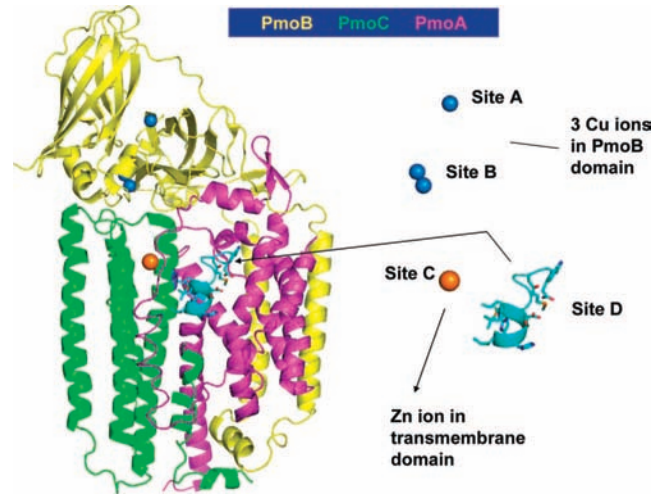


FIGURE 3. Architecture of pMMO according to the X-ray crystal structure of Lieberman and Rosenzweig.[17]

catalytic machinery of the enzyme, wherein one of the two tricopper clusters supported both dioxygen chemistry and alkane hydroxylation and the other dioxygen chemistry only. It was evident that only the first cluster needed to be a triad of copper atoms to support the oxo-transfer chemistry; for the second grouping of copper ions to support dioxygen chemistry, a dinuclear site and a mononuclear copper would suffice.

Evidence for at least one tricopper cluster in pMMO came originally from low-temperature EPR of isolated membrane fragments enriched in pMMO or purified pMMO reconstituted in detergent micelles,[27] when the enzyme was handled in the absence of hydrocarbon substrate. The 4 K EPR typically consisted of a superposition of signals: a type 2 Cu^{II} signal at $g_{av} \approx 2.12$ with Cu hyperfine in the parallel region ($g_{||} = 2.24$) and ^{14}N superhyperfine in the perpendicular region ($g_{\perp} = 2.059$) and an almost featureless isotropic signal centered at $g \approx 2.1$ that we assigned to a trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster (Figure 2.) The total intensity of the EPR signals corresponded to about 2 Cu^{II} ions, consistent with one type 2 Cu^{II} site and a ferromagnetically exchange-coupled $Cu^{II}Cu^{II}Cu^{II}$ cluster with EPR transitions from only the $S = {}^3/_2$ ground state.[28] Unlike the type 2 Cu^{II} signal, the cluster signal did not saturate at high microwave power.[27,29]

These observations could be readily understood in terms of the dioxygen chemistry proposed for the C-clusters. When these six copper ions are turned over in the presence of hydrocarbon substrate, there is perfect matching of the number of reducing equivalents at the active site (including the two reducing equivalents coming from the hydrocarbon substrate) and the number of oxidizing equivalents from the two dioxygen molecules participating in the dioxygen chemistry. On the other hand, there is an overall mismatch in the number of reducing and oxidizing equivalents at the active site when there is no substrate to provide the two extra reducing equivalents to complete the oxidative phase of the catalytic cycle.[1] In the latter scenario, the dioxygen chemistry at the "hydroxylation" tricopper cluster will be accompanied by transfer of one reducing equivalent from the mononuclear copper of the second "cluster" to produce the $Cu^{II}Cu^{II}Cu^{II}$ cluster EPR and the type 2 Cu(II) signal with the ^{14}N superhyperfine. Reaction of the second dioxygen with the dinuclear copper site of the second "cluster" would yield the bis(μ-oxo) dicopper(III) or a (μ-η^2:η^2-peroxo) dicopper(II) cluster species, which is EPR silent.

Since all 15 copper ions in the purified pMMO have been shown to participate in redox, dioxygen chemistry, and hydrocarbon oxidation, it seems unlikely that the putative diiron center implicated in the purified enzyme with the 10% occupancy is directly involved in the hydroxylation of alkane. There is no evidence that these irons participate in redox and dioxygen-turnover chemistry in the first place. If there is a role for the diiron center in pMMO, it could be an auxiliary one, for example, enhancing the activity of the copper enzyme in the 10% of the proteins containing it.

Three-Dimensional Structure

The crystal structure of pMMO was reported by the laboratory of Amy Rosenzweig in 2005.[17] According to this work, the protein crystallizes as a trimer of $\alpha\beta\gamma$ monomers with three copper ions and one zinc ion per monomer. A ribbon diagram of the structure is shown in Figure 3, with different colors used to depict the three subunits. PmoA and PmoC are mostly transmembrane, each with approximately six transmembrane segments. The N- and C-terminal subdomains of PmoB are exposed to the cytosol and are anchored at the

TABLE 1. Repeat of the Purification of pMMO According to the Procedures of Lieberman et al.[13,17] and Comparison of the Specific Activity and Metal Contents Obtained with Those Previously Reported for the Protein Preparation Used in the Crystallographic X-ray Analysis

	specific activity (nmol/min/mg protein)		copper content (atoms/protein)		iron content (atoms/protein)	
pMMO sample, status of purification	this work	refs 13 and 17	this work	refs 13 and 17	this work	refs 13 and 17
membranes	88.9	19.0	12−15	*a*	0.9	*a*
membrane fragments solubilized in detergent	93.5	3.9	13.3	10.4	*b*	1.1
purified detergent−protein complex (prior to $(NH_4)_2SO_4$ precipitation)	21.5	*a*	10.0	*a*	*b*	*a*
purified detergent−protein complex (following $(NH_4)_2SO_4$ precipitation, detergent resolubilization, and anion exchange chromatography)	*c*	*c*	2.5	2.4	*b*	0.8

[a] No data provided or available. [b] Not detected. [c] Inactive.

water−membrane interface by two α-helical transmembrane segments inserted into the membrane. This protein architecture is consistent with the membrane topology predicted previously by protease digestion of membrane fragments followed by mass-fingering of the peptides released by MALDI-TOF-MS.[1]

The three copper ions and the zinc ion reported in the crystal structure are also shown in Figure 3, with the three copper ions at site A and site B in blue and the zinc ion at site C in brown. Highlighted in the transmembrane domain of the crystal structure at site D is a cavity consisting of a hydrophilic cluster of potential metal-ligating residues, including His38, Met42, Met45, Asp47, Trp48, Asp49, and Glu100 from PmoA and Glu154 from PmoC. While this "cluster of hydrophilic residues" was previously discounted as a metal-binding site,[17] our view was that without counterions to balance the charge of the hydrophilic residues, the electrostatic energy of sustaining this cavity would be extremely high.

We surmise that metal ions must have been stripped away from site D during the purification of the protein for crystallographic analysis.[16] Repeat of the purification procedures adopted by the Northwestern group indicated that as many as 12 of the ca. 15 copper ions were removed from the protein during the ammonium sulfate fractionation with concomitant loss of enzymatic activity (Table 1.) We henceforth refer to the X-ray structure as the Cu_3-pMMO. With essential cofactors missing, it would be inappropriate to derive conclusions on the catalytic cycle and the mechanism of hydroxylation of alkanes from the X-ray structure.[18,21]

Evidence for a Tricopper Cluster in pMMO

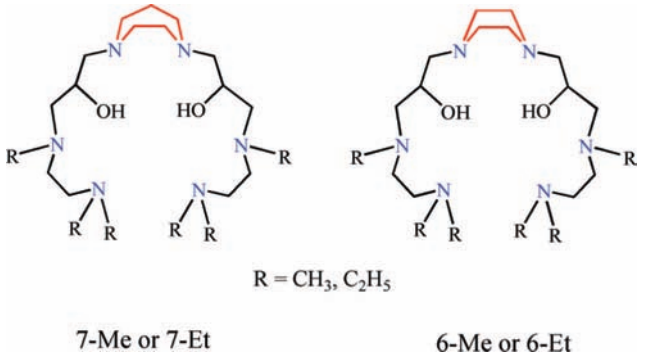
Based on the X-ray structure, Rosenzweig and co-workers[17,18] have ruled out the possibility of a tricopper cluster in the enzyme. We have now obtained new data to reinforce our view that this tricopper cluster is missing from the X-ray structure.

First, the isotropic EPR signal attributed to the putative $Cu^{II}Cu^{II}Cu^{II}$ cluster have now been resolved from the type 2 Cu^{II} signal by redox potentiometry/EPR.[16] These copper sites have distinct redox potentials, and thus by titration of the protein at different cell potentials in an electrochemical cell, the spectroscopic features for each site could be distinguished and individually assigned. As an example, the intensity of the signal at +53 mV (versus SHE) corresponded to approximately two copper ions per protein monomer, as expected for contributions from one type 2 Cu^{II} site and one $Cu^{II}Cu^{II}Cu^{II}$ cluster (Figure 2). With increasingly more negative cell potentials, the type 2 Cu^{II} EPR decreased gradually in intensity, beginning at +18.3 mV. At −121.3 mV, the spectrum eventually gave way to an isotropic signal centered at $g = 2.05$ (intensity corresponding to 0.13 $Cu^{II}Cu^{II}Cu^{II}$ cluster). These results provide unequivocal evidence for a tricopper cluster in our preparation of pMMO.

Second, the essentially identical EPR has also been recorded for several model ferromagnetically coupled trinuclear $Cu^{II}Cu^{II}Cu^{II}$ clusters designed and synthesized in this laboratory.[30] These tricopper complexes are based on the ligands (L) 3,3′-(1,4-diazepane-1,4-diyl)bis(1-((2-(dimethylamino)ethyl)(methyl)amino)propan-2-ol) (**7-Me**) or 3,3′-(1,4-diazepane-1,4-diyl)bis(1-((2-(diethylamino)ethyl)(ethyl)amino)-propan-2-ol (**7-Et**) (Scheme 1), which contain six neutral amines and two hydroxyl groups that are capable of trapping three copper ions simultaneously. $[Cu^ICu^ICu^I(L)]^{1+}$ complexes were readily prepared by treating 3 equiv of $Cu[(CH_3CN)_4](X)$ ($X = ClO_4^-$ or BF_4^-) in anhydrous CH_3CN solution with one equivalent of [**7-Me**]$^{2-}$[Na_2]$^{2+}$ or [**7-Et**]$^{2-}$[Na_2]$^{2+}$. Oxygenation of the $[Cu^ICu^ICu^I(L)]^{1+}$ complexes yielded a deep blue $[Cu^{II}Cu^{II}Cu^{II}(L)(O)]^{2+}$ species with X-ray structure and 4 K EPR shown in Figure 4A,B, respectively.

Interestingly, these $[Cu^ICu^ICu^I(L)]^{1+}$ complexes reacted with dioxygen to form an intermediate capable of facile O-atom

SCHEME 1. Ligands Used To Construct the $[Cu^{I}Cu^{I}Cu^{I}(L)]^{1+}$ Complexes



insertion across the central C−C bond of benzil and 2,3-butanedione at ambient temperature and pressure. These complexes also catalyzed facile O-atom transfer to the C−H bond of CH_3CN to form glycolonitrile. (Figure 5) These observations are significant because they illustrate that an appropriately designed trinuclear $Cu^{I}Cu^{I}Cu^{I}$ cluster, upon activation with dioxygen, can mediate facile O-atom transfer to organic substrates similar to the chemistry catalyzed by pMMO to simple alkanes. An important difference is that our model tricopper complexes form weak transient complexes with acetonitrile, benzil, and 2,3-butanedione, whereas they do not have comparable affinity for hydrocarbon substrates. Nevertheless, preliminary evidence for the hydroxylation of hexane was also suggested.[30]

Rebuilding Three Copper Ions into Site D in the Crystal Structure

Assuming that the overall fold of the Cu_3-pMMO is not dramatically compromised by the loss of the remaining copper ions, we have made an attempt to rebuild copper ions back into the protein scaffold.[16] This effort has led to a tricopper cluster at site D (Figure 6). A trinuclear $Cu^{II}Cu^{II}Cu^{II}$ cluster capped with a μ-oxo was computationally modeled into the site taking into account favorable side-chain rotomers, potential hydrogen bonding interactions, and metal−ligand bond and angle geometries and optimizing the geometry of the residues and the metal ions to minimize the energy of the modeled tricopper site. The coordinated ligands and the geometry of the cluster, including the Cu−Cu and Cu−O distances, are all reasonable demonstrating the feasibility of pMMO to accommodate a tricopper cluster. The ligands to the copper atoms in the model are as follows: PmoC Glu154 and PmoA His38 for Cu1; PmoA Met42 and Asp47 for Cu2; and PmoA Asp49 and Glu100 for Cu3. The carbonyl of PmoA Ala41 refines to a position where it can also bind Cu2. The trinuclear $Cu^{II}Cu^{II}Cu^{II}$ structure modeled here would correspond to that of the fully oxidized cluster after turnover by dioxygen in the absence of hydrocarbon substrate.

The electrostatic energy of the protein should be significantly reduced by rebuilding the tricopper cluster into site D. The oxidized tricopper cluster with the capped O^{2-} is electrically neutral. When the cluster is reduced, the site bears a charge of −1 only. Accordingly, it is unreasonable for the cavity at site D to harbor the hydrophilic cluster without the copper ions.

The Search for the Substrate Binding Site

Given that the oxidation of hydrocarbons mediated by pMMO involves a direct concerted O-atom insertion mechanism, there must be a binding pocket for the hydrocarbon substrate in close proximity to the catalytic site. This pocket must be capable of accommodating only the limited number of substrates known to be oxidized by the enzyme. It follows then that if the function of one of the copper sites (sites A or C, B, and D) is to mediate the O-atom transfer from the copper ions to the hydrocarbon substrate, the hydroxylation site should embrace both this copper site and the hydrophobic cavity for the hydrocarbon substrate. In the concerted mechanism, the "O" atom harnessed by the copper complex must enter into a transition state with the C−H bond that is being activated.

Accordingly, we searched for possible binding site(s) for the hydrocarbon substrate within the three-subunit $\alpha\beta\gamma$ monomer and used this information to home in on the oxidation site. Pentane was selected as the ligand to search for possible binding site(s) in the receptor protein based on the substrate specificity of pMMO. The search was carried out using the binding sites prediction program Dockligand (LigandFit) on Discovery Studio 1.7 (Accelrys Software Inc.). Pentane was chosen because it represented the substrate with the largest surface area and volume that could be oxidized by the enzyme. Apparently, all substrates of pMMO use the same binding pocket, including acetylene, the suicide substrate that modifies His38 of PmoA at site D.

We first subjected the published crystal structure to the Global Protein Surface Survey (GPSS) analysis on the GPSS Web site, http://gpss.mcsg.anl.gov. The GPSS PyMOL plugin was applied to the pMMO protein monomer (PDB ID WS_1YEW1), which was constructed from the PDB model of 1YEW. The calculations yielded 122 CASTp surfaces, but on the basis of the surface area and volume of the cavity required to accommodate pMMO substrates, the most probable site was determined to be the hydrophobic pocket previously identified near site D in the structure adjacent to the tricopper cluster that had been modeled into the crystal structure (Figure 7A).

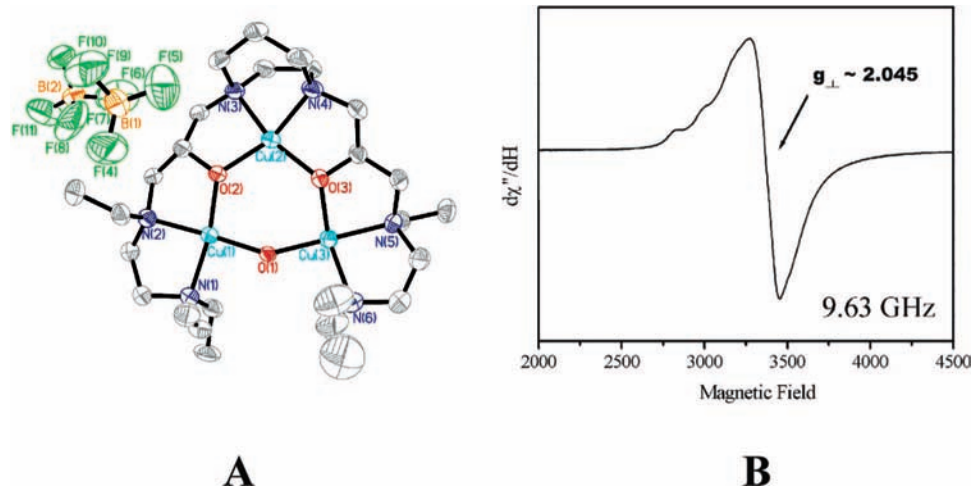


FIGURE 4. (A) ORTEP representation of $[Cu^{II}Cu^{II}Cu^{II}(\mathbf{7Et})(O)]^{2+}(BF_4^-)_2$; (B) 4 K EPR spectrum of $[Cu^{II}Cu^{II}Cu^{II}(\mathbf{7\text{-}Et})(O)]^{2+}(BF_4^-)_2$ in CH_3CN.

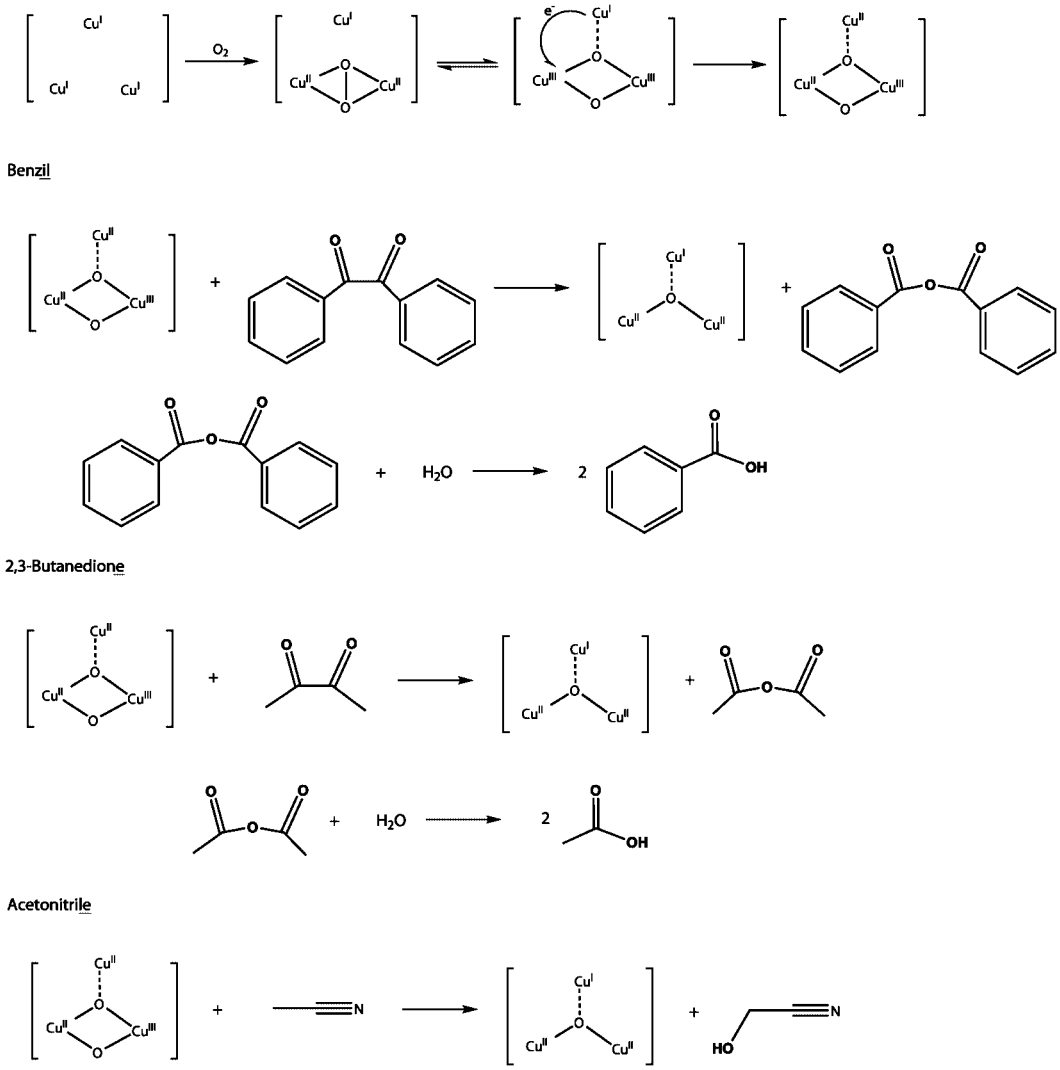


FIGURE 5. "Singlet oxene" insertion into C−C bonds (benzil and 2,3-butanedione) and a C−H bond (acetonitrile) mediated by $[Cu^ICu^ICu^I(L)]^{1+}$ complexes upon activation by dioxygen.

In support of the GPSS analysis, the same binding site was also predicted by Dockligand (LigandFit). This putative hydrophobic pocket was sufficiently long to bind only C1−C5 hydrocarbons, and it was wide enough to accommodate only

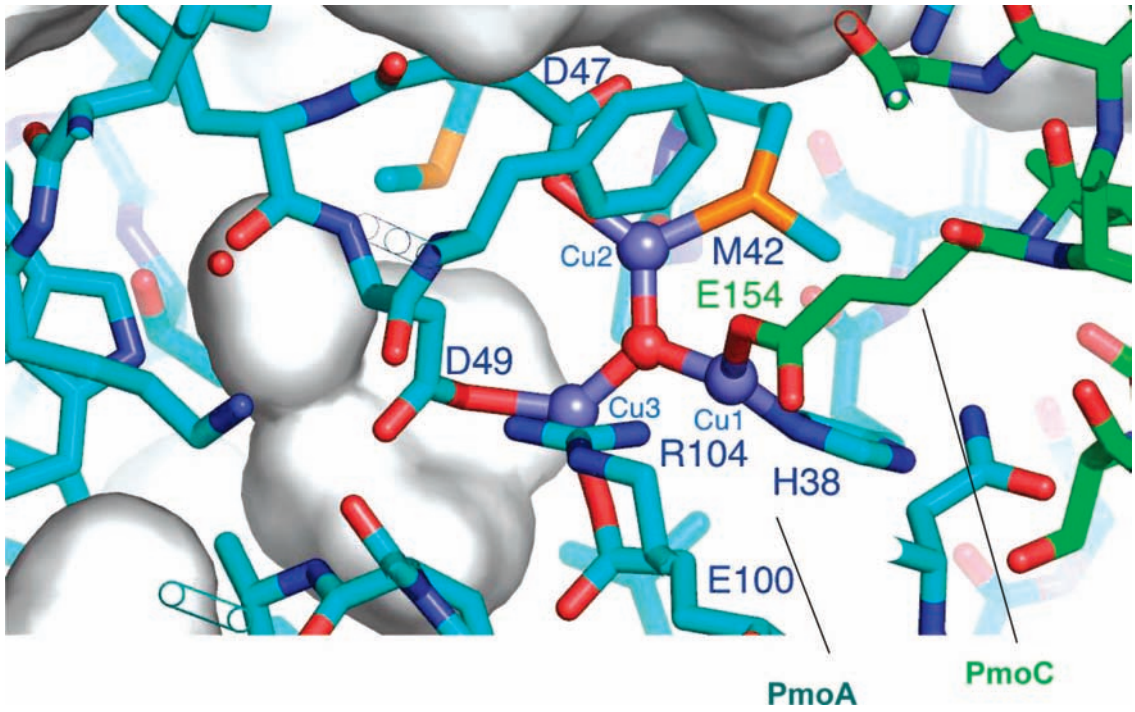


FIGURE 6. Tricopper cluster modeled as $Cu^{II}Cu^{II}Cu^{II}$ with capping "oxo" at site D of the crystal structure. The amino acid side chains coordinating the three copper ions are as follows: Cu1, PmoA His38 and PmoC Glu154; Cu2, PmoA Asp47 and Met42; Cu3, PmoA Asp49 and Glu100.

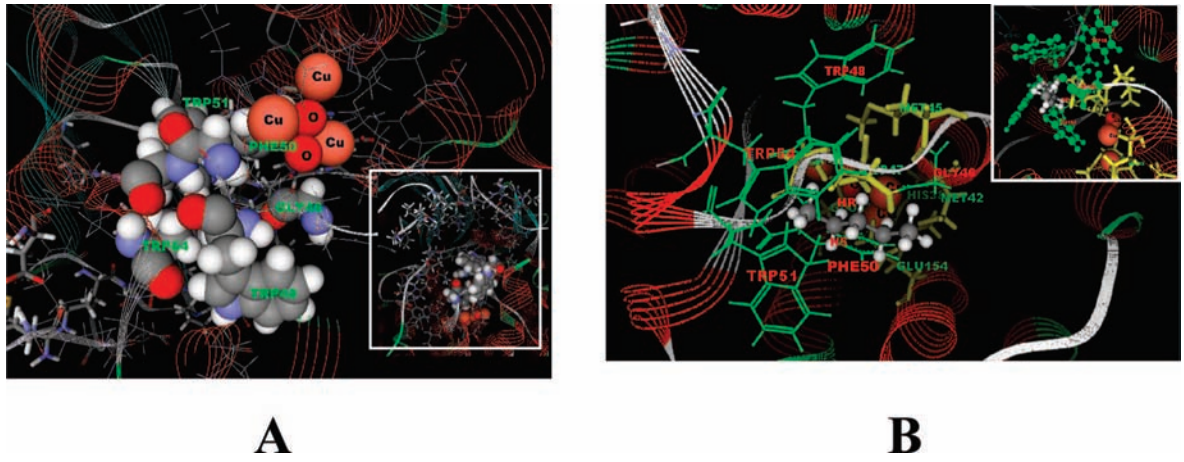


FIGURE 7. (A) The hydrophobic pocket adjacent to the site of the putative tricopper cluster (site D), with the amino acid residues forming the cavity highlighted by CPK models—inset, the most probable CASTp surface calculated from the GPSS program; (B) Discovery Studio modeling of the pentane molecule within the hydrophobic pocket (lined by Gly46, Trp48, Phe50, Trp51, and Trp54), showing an orientation of the hydrocarbon (side view) with the H_R atom of the C2 carbon directed at site D of pMMO—inset, an end view of the site showing an orientation of the activated oxygen of the tricopper cluster directed at C2—H_R of the pentane. The amino acid residues associated with the hydrophobic cavity are denoted in green, and those associated with the tricopper cluster in site D are denoted in yellow.

straight-chain alkanes. The hydrophobic "channel" was lined by the aromatic residues Trp48, Phe50, Trp51, and Trp54 of PmoA, the 28-kDa subunit, and was "closed" at one end (Figure 7B). Located at the open substrate entrance to the pocket was Gly46. With the "probing" hydrocarbon substrate fully inserted into the pocket, the putative tricopper cluster was directed at the secondary carbon of the substrate near the depth of the pocket, perfectly poised for O-atom transfer to this secondary carbon when the tricopper cluster was activated by dioxygen. In this molecular model, the H_R of the C2 carbon was directly facing site D (side view; inset, end view). Concerted O-atom insertion from a dioxygen-activated tricopper cluster at site D would lead to the formation of the (2*R*)-pentan-2-ol. Reorientation of the pentane molecule within the same pocket would subject the H_S of the C2 carbon toward site D. In this configuration, O-atom transfer would result in

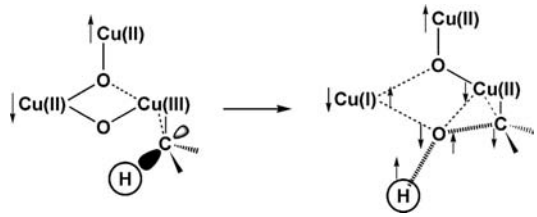


FIGURE 8. Details of the adiabatic "singlet oxene" transfer from a dioxygen activated tricopper cluster to methane to form the transition state. The ↑ and ↓ denote "up" and "down" directions of the unpaired electron spins. (Reproduced from ref 1).

(2*S*)-pentan-2-ol as the product. Experimentally, only (2*R*)-pentan-2-ol and (2*S*)-pentan-2-ol were observed (ee = 80%).[2]

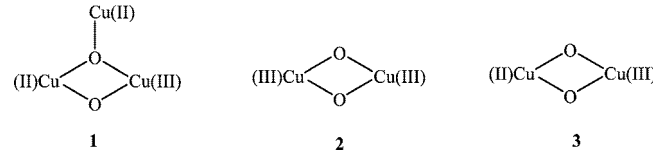
The above analysis argues for a hydrophobic substrate-binding site in pMMO located within 6 Å of site D of the crystal structure adjacent to the site of the tricopper cluster that has been implicated in the catalytic chemistry. Sites A, B, and C are, respectively, ~40, 26, and 13 Å away from the hydrophobic pocket identified here, too far for direct concerted O-atom insertion into a C−H bond.[16,17,21]

The O-Atom Transfer Process in pMMO

We have conjectured that the "active" species that promotes O-atom insertion in pMMO is a mixed-valence $[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$ intermediate formed following reaction of a reduced trinuclear $[Cu^{I}Cu^{I}Cu^{I}]^{3+}$ cluster with dioxygen.[1] The main features of this mechanism are illustrated in Figure 8. Although only two of the three reducing equivalents are required for hydroxylation chemistry, the third reducing equivalent in the hydroxylation cluster facilitates the efficient O-atom transfer based on the concerted "singlet oxene" insertion into the C−H bond.[20]

In principle, O-atom transfer to a C−H bond could also be mediated by a dinuclear copper cluster.[19,21,31] Activation of a $Cu^{I}Cu^{I}$ complex by dioxygen could lead to a bis(μ-oxo)Cu^{II}-$_{I}Cu^{III}$ species, the structurally related (μ-η^2:η^2-peroxo)$Cu^{II}Cu^{II}$, or (hydroperoxo)$Cu^{II}Cu^{II}$ species. A "singlet oxene" transfer from the bis(μ-oxo)$Cu^{III}Cu^{III}$ species to a hydrocarbon substrate could take place when one of the bridging μ-oxos is in a position to form an appropriate transition state complex with the C−H bond. In fact, the chemistry proceeds on a "singlet" reaction potential surface without spin crossover just as in the case of a mixed-valent $[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$. Most bis($\mu$-oxo)$Cu^{III}Cu^{III}$ species are, however, inert toward this process.[31] Density functional theory (DFT) calculations indicate that it takes considerable stretching of the two Cu−O bonds to reach the transition state, so the kinetic barrier is significantly higher than in the case of the mixed-valent $[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$ species.[20]

SCHEME 2. Three Possible Models of the Activated pMMO Active Site Used in the DFT Calculations



1 **2** **3**

Direct O-atom transfer from a dicopper complex to an exogenous ligand is rare. More likely, the activated dicopper complex is degraded internally via intramolecular proton-coupled electron transfer from the ligand to reduce the dicopper cluster, followed by •OH radical recombination to the ligand oxidized. Indeed, if the bis(μ-oxo)$Cu^{III}Cu^{III}$ is reductively activated by an external reducing equivalent, the complex becomes significantly more reactive toward exogenous substrates.[20] But then the O-atom transfer occurs via hydrogen atom abstraction followed by radical recombination, and the stereochemistry of the process is dramatically different from the concerted direct O-atom insertion highlighted above.

Theoretical analysis of the overall problem, indeed, indicates that the putative tricopper cluster offers a significantly more facile pathway for alkane hydroxylation compared with the traditional or mixed-valent dicopper cluster. Chen and Chan[20] have carried out DFT calculations directed toward elucidation of the C−H bond activation mechanisms that might be adopted by pMMO in the case of methane. Three of the most probable models were considered: (i) the trinuclear $[Cu^{II}$-$Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$ species **1** proposed by Chan et al.,[1] (ii) the most frequently used model, the bis(μ-oxo)$Cu^{III}Cu^{III}$ complex **2**, and (iii) the mixed-valent bis(μ-oxo)$Cu^{II}Cu^{III}$ complex **3** (Scheme 2). First-order rate constants at 300 K for the O-atom transfer step based on the kinetic barrier for the formation of the transition state in the case of complexes **1** and **2** and for the rate-limiting hydrogen abstraction step for complex **3** are summarized in Table 2. It is evident from these results that the trinuclear $[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$ complex **1** offers the most facile pathway, and it yields a KIE (k_H/k_D) in agreement with experiment. The KIEs (~5) associated with the concerted oxene-insertion process mediated by both complexes **1** and **2** are dramatically different from the value of ca. 50 predicted for the direct hydrogen atom abstraction chemistry mediated by complex **3**. Since these kinetic isotope effects include quantum mechanical tunneling corrections, they may be compared directly with experiment. It would seem that these results further bolster our case for a tricopper cluster in pMMO.

TABLE 2. First-Order Rate Constants and KIE Values Predicted for the Hydroxylation of Methane by the Trinuclear $[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$ Complex, the Dinuclear Bis(μ-oxo)$Cu^{III}Cu^{III}$ Complex, and the Dinuclear Mixed-Valent Bis(μ-oxo)$Cu^{II}Cu^{III}$ Complex According to Density Functional Theory Calculations

	T, K	$k(CH_4)^a$, s^{-1}	$k(CD_4)^b$, s^{-1}	k_H/k_D
		$[Cu^{II}Cu^{II}(\mu\text{-}O)_2Cu^{III}]^{3+}$		
facile	300	2.91×10^4	6.83×10^3	4.3 (5.2)[c]
		bis(μ-oxo)$Cu^{III}Cu^{III}$		
inert	300	5.67×10^{-1}	1.49×10^{-1}	3.8 (4.9)[c]
		bis(μ-oxo)$Cu^{II}Cu^{III}$		
slow	300	1.19×10^2	7.65	16 (49)[c]

[a] Rate constant for CH_4 as substrate. [b] Rate constant for CD_4 as substrate. [c] Tunneling effects are included.

Summary

We believe important strides have been made toward understanding the structure and function of pMMO with the recent crystal structure of the Cu_3-enzyme and the efforts that we have made toward rebuilding the missing copper ions into the protein structure in order to reconcile the X-ray crystal structure with the wealth of biochemical and biophysical data compiled over the past two decades. Specifically, we believe that we have made a strong case for a tricopper cluster in pMMO and have delineated the role of this novel structure in mediating facile O-atom transfer to organic substrates. This tricopper cluster, together with the mononuclear and dinuclear copper cofactors seen in the crystal structure, presumably accounts for the six copper ions required for the turnover of the enzyme. At the very least, a minimal structural framework is now in place, and we can begin to address a number of issues relating to the structure and function of this interesting enzyme.

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Steve S.-F. Yu grew up in Taiwan. He graduated from National Tsing Hua University, Taiwan, with a B.S. degree in chemistry in 1991 and a Ph.D. in 1998. He is currently an Assistant Research Fellow in the Institute of Chemistry at Academia Sinica, Taiwan. His research program focuses on biocatalysis mediated by metallo-enzymes in microbial cells and membranes.

FOOTNOTES

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