Methane Monooxygenase

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One is Lonely and Three is a Crowd: Two Coppers Are for Methane Oxidation**

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Mild activation of inert C–H bonds attracts increasingly feverish research efforts as the global predicament of dwindling resources grows increasingly dire. The desirability of methane as a chemical feedstock presents itself in this context: Functionalization of this simplest hydrocarbon would unlock the world's reserves of natural gas for the synthesis of necessary chemicals from this abundant C_1 building block. However, with a C–H bond strength of 104 kcal mol^{-1}, methane possesses the most difficult hydrocarbon bond to oxidize. Current industrial methods for converting methane into useful chemical products incur prohibitive energy costs and lack selectivity.[1]

Not so for nature's methane monoxygenases, the enzymes utilized in the metabolism of CH_4 by bacteria that use methane as a primary energy and biosynthetic material source. Two enzyme classes, soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), selectively oxidize CH_4 to CH_3OH at ambient temperature and pressure. The well-understood sMMO employs an active-site diiron cluster to bind and activate dioxygen for this two-electron oxidation. Extensive bioinorganic research over several decades has successfully elucidated the probable mechanistic pathways for sMMO catalysis.[2]

By contrast, the transmembrane protein pMMO has been recalcitrant in yielding the secrets of its structure and reactivity. A copper ion was implicated as the key cofactor in this metalloenzyme, but clues to its role in pMMO had proven inscrutable. For example, measurements of pMMO copper loading and "per copper ion" oxidation activity varied.[3]

Three main hypotheses have arisen concerning the pMMO active site: 1) at least one, but possibly multiple, trinuclear copper (Cu_3) clusters as loci of electron transfer and catalytic reactivity,[2b] 2) a diiron active site,[4] and 3) a dicopper center.

That last proposal rose to the forefront with the publication of the enzyme's first X-ray crystal structure (Figure 1).[5] Although a major breakthrough, this fruit of Rosenzweig and Lieberman's efforts has raised more questions than it has answered. Is the dicopper center indeed the catalytic active site? A single copper site found in the first X-ray structure was absent from a second structure determined from a pMMO from a different species:[6] does it have any role? The third site, occupied by zinc in the first structure, was replaced by copper in the second: is this the active site?

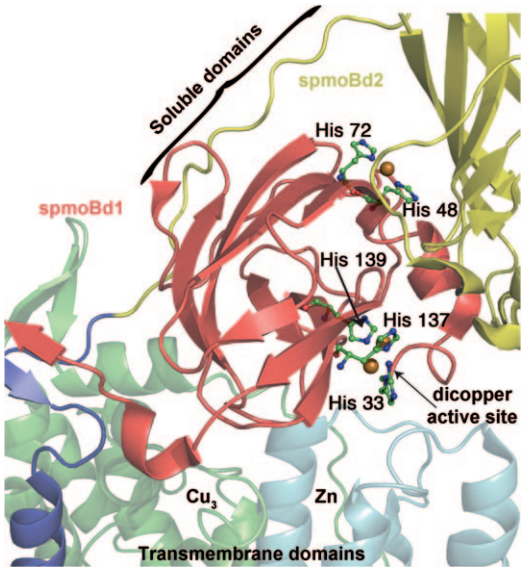

Figure 1. The pMMO (*M. capsulatus* (Bath)) structure. Soluble domains from subunit B and transmembrane regions consisting of a portion of subunit B (blue) and subunits A (faint green) and C (faint cyan) are shown. Studies on recombinant proteins consisting of only the soluble domains, spmoBd1 (red) and/or spmoBd2 (yellow), demonstrate the dicopper center is the active site of pMMO. PDB code: 1YEW.

In fact, the residues in the "zinc" site recapitulate the binding motif of diiron sites in iron monooxygenases, including sMMO. Some researchers argue that the purification/crystallization methods have stripped a diiron active site from this location. Furthermore, a cluster of hydrophilic residues in

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one of the transmembrane domains could provide the ligands for a trinuclear copper cluster at that site, as modeled theoretically (Figure 1).[3b,7] Finally, further doubt that the X-ray structures truly reflect pMMO cofactors stems from the inactivity of crystallized proteins.

These problems required a fresh line of attack. The most recent results from Amy Rosenzweig's lab provide it.[8] The authors have used recombinant DNA to express, in *E. coli,* modified pmoB protein from *Methylococcus capsulatus* (Bath), which is the subunit sequence incorporating the solvent-exposed portion of the enzyme that houses the two copper sites (mononuclear and dinuclear). Replacement of the two membrane-spanning helices in the full subunit with a short amino acid sequence renders the recombinant proteins soluble without detergent (spmoB). Thus, the authors took an inspired approach to pMMO by peeling back one layer of the onion to see what the layer itself could tell about the whole.

The structure and reactivity of copper-loaded recombinant proteins were then investigated. Recombinant spmoB binds on average three copper ions, as predicted from the full crystal structure. X-ray absorption and extended X-ray absorption fine structure (EXAFS) spectroscopic analysis revealed a short Cu–Cu interaction, as for the full enzyme[5,9]—evidence for the dicopper site. Furthermore, in using recombinant DNA for the subunit, the authors could carry out mutagenesis studies, which had been impossible with the full enzyme. Mutating any of the three histidine binding residues in the dicopper site (Figure 2) lowered the

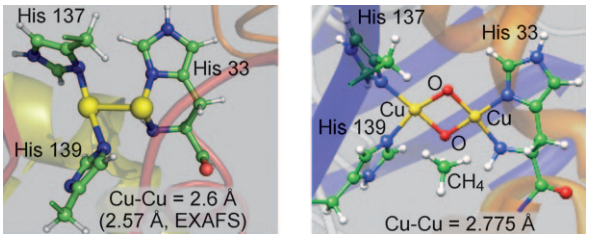


Figure 2. Left: The dicopper active site as determined from the low-resolution pMMO X-ray structure (PDB code: 1YEW). Right: From Yoshizawa and co-workers,[10] a computationally derived mixed-valent Cu^{II}–Cu^{III} bis-μ-oxo reactant complex for methane hydroxylation.

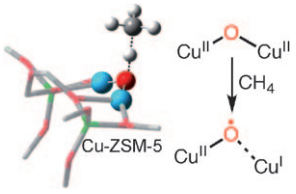
copper ion uptake of the protein and eliminated the copper–copper interaction from the EXAFS data.[5]

Most strikingly, spmoB, with its three copper ions, oxidizes methane. The spmoBd2 domain, which lacks metal-binding sites, and mutant spmoB, in which two of the dicopper site binding residues are replaced by alanine, do not. Mutation of the amino acid ligands to the mononuclear copper attenuates, but does not eliminate, reactivity. These results inexorably lead to two conclusions: 1) The pMMO active site is in the pmoB subunit, and thus not in either site where a diiron or trinuclear copper cluster is proposed to reside; and 2) a dicopper center is responsible for the catalytic oxidation of methane.

That the experiments with the recombinant subunit are relevant to the full enzyme is demonstrated by elegant studies with as-isolated pMMO. Cyanide treatment removes all metal content from membrane enzyme and consequently abrogates its oxidative activity. Subsequent titration of stoichiometric copper into such apo pMMO samples restores 90% of the methane oxidation activity at 3 equiv Cu (per pMMO protomer). Higher copper loadings start to inhibit pMMO, indicating that additional copper sites (such as multiple trinuclear clusters) are not required for activity. Furthermore, addition of iron—in the presence or absence of copper—conferred no oxidative activity to the protein.

These results with as-isolated pMMO would appear to vanquish alternate hypotheses for the active site of pMMO. The minimal uptake of iron and the complete lack of activity in either as-isolated pMMO or the recombinant proteins reconstituted with iron rules out a diiron active site. And the fact that pMMO achieves maximum activity with three copper ions (with those ions traced by EXAFS to the mononuclear and dinuclear copper sites) disfavors the presence of a trinuclear copper cluster(s) in the enzyme. The recombinant protein does not precisely mimic pMMO activity, as its rate of methane oxidation is significantly lower than the full enzyme. However, the possible implication that other metal sites (either catalytic or electron transfer) must contribute substantially to pMMO seems to be ruled out by the metal titration results.

In conclusion, the dicopper center is thus the pMMO active site (Figure 2). Researchers must begin to address the more intriguing question: How does a dinuclear copper center oxidize methane to methanol? Studies with the pMMO enzyme itself have yielded few mechanistic clues. But, as both experimental and especially computational chemists have entered the pMMO fray, tantalizing hypotheses have been proposed. Calculations by Yoshizawa and co-workers suggest that a one-electron reduced $Cu^{II}Cu^{III}(\mu\text{-}(O)_2)$ species has greater oxidizing power than either a symmetric $Cu^{III}{}_2(\mu\text{-}(O)_2)$ or $Cu^{II}{}_2(O_2{}^{2-})$ (that is, peroxo) congener—great enough to cleave the methane C–H bond (Figure 2).[10] Chan and co-workers also invoke such a mixed-valent dicopper methane oxidizer, although as a portion of a tricopper moiety.[3b,11] In a recent spectroscopic–computational study on a copper-loaded zeolite (Cu ZSM-5) that oxidizes methane, Solomon, Schoonheydt and co-workers demonstrated that a relatively simple Cu^{II}–O–Cu^{II} moiety is the active oxygenating agent.[12] Similar to the pMMO theoretical results, the zeolite oxodicopper(II) site can, according to DFT calculations, develop oxygen-centered radical character (to abstract a hydrogen atom from methane (Scheme 1).



Scheme 1. Calculated transition state for the oxodicopper(II) methane-oxidizing Cu ZSM-5 zeolite.[12] Can such an intermediate be considered for the the pMMO dicopper catalytic center? If so, how would it form from enzymatic copper(I)–dioxygen chemistry?

The mixed-valent oxodicopper(II/III) or simple oxodicopper(II) centers are both candidates for the enzyme active-site methane oxidizer. The former has not yet been identified or isolated among the known crop of Cu_2O_2 synthetic model compounds;[1c,13,14] the latter has some precedent,[15] but does not have a developed chemistry. Suggestively, several reports of copper-promoted oxidation of strong hydrocarbon bonds suggest that reduction of a Cu_2O_2 core to a putative $Cu^{II}Cu^{III}$ mixed-valent system precedes reactivity.[13] This raises another crucial under-addressed question: How does the dicopper site form a methane-oxidizing species by reaction with dioxygen and reducing equivalents? And where do those electron equivalents come from: From other copper ion sites, or perhaps from neighboring amino acid residues?[10]

The results from the Rosenzweig laboratory appear to settle the question of what is the pMMO active site, yet many questions and challenges remain before a comprehensive understanding of copper-mediated methane oxidation is achieved.

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[1] a) H. Arakawa et al., *Chem. Rev.* **2001**, *101*, 953; b) B. L. Conley, W. J. Tenn III, K. J. H. Young, S. K. Ganesh, S. K. Meier, V. R. Ziatdinov, O. Mironov, J. Oxgaard, J. Gonzales, W. A. Goddard III, R. A. Periana, *J. Mol. Catal. A* **2006**, *251*, 8; c) R. A. Himes, K. D. Karlin, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18877.

[2] a) B. J. Wallar, J. D. Lipscomb, *Chem. Rev.* **1996**, *96*, 2625; b) M. Merkx, D. A. Kopp, M. H. Sazinsky, J. L. Blazyk, J. Müller, S. J. Lippard, *Angew. Chem.* **2001**, *113*, 2860; *Angew. Chem. Int. Ed.* **2001**, *40*, 2782; c) M.-H. Baik, M. Newcomb, R. A. Friesner, S. J. Lippard, *Chem. Rev.* **2003**, *103*, 2385; d) I. Siewert, C. Limberg, *Chem. Eur. J.* **2009**, *15*, 10316.

[3] a) A. S. Hakemian, A. C. Rosenzweig, *Annu. Rev. Biochem.* **2007**, *76*, 223; b) S. I. Chan, S. S.-F. Yu, *Acc. Chem. Res.* **2008**, *41*, 969.

[4] M. Martinho, D. W. Choi, A. A. DiSpirito, W. E. Antholine, J. D. Semrau, E. Munck, *J. Am. Chem. Soc.* **2007**, *129*, 15783.

[5] R. L. Lieberman, A. C. Rosenzweig, *Nature* **2005**, *434*, 177.

[6] A. S. Hakemian, K. C. Kondapalli, J. Telser, B. M. Hoffman, T. L. Stemmler, A. C. Rosenzweig, *Biochemistry* **2008**, *47*, 6793.

[7] S. I. Chan, V. C.-C. Wang, J. C.-H. L. Lai, S. S.-F. Yu, P. P.-Y. Chen, K. H.-C. Chen, C.-L. Chen, M. K. Chan, *Angew. Chem.* **2007**, *119*, 2038; *Angew. Chem. Int. Ed.* **2007**, *46*, 1992.

[8] R. Balasubramanian, S. M. Smith, S. Rawat, L. A. Yatsunyk, T. L. Stemmler, A. C. Rosenzweig, *Nature* **2010**, *465*, 115.

[9] R. L. Lieberman, K. C. Kondapalli, D. B. Shrestha, A. S. Hakemian, S. M. Smith, J. Telser, J. Kuzelka, R. Gupta, A. S. Borovik, S. J. Lippard, B. M. Hoffman, A. C. Rosenzweig, T. L. Stemmler, *Inorg. Chem.* **2006**, *45*, 8372.

[10] a) Y. Shiota, K. Yoshizawa, *Inorg. Chem.* **2009**, *48*, 838; b) K. Hori, Y. Shiota, K. Yoshizawa in *Wiley Series on Reactive Intermediates in Chemistry and Biology*, *Vol. 5 (Series* Ed.: S. E. Rokita), *Copper-Oxygen Chemistry* (Eds.: S. Itoh, K. D. Karlin), Wiley-VCH, Weinheim, **2010**, in press.

[11] P. P. Y. Chen, S. I. Chan, *J. Inorg. Biochem.* **2006**, *100*, 801.

[12] J. S. Woertink, P. J. Smeets, M. H. Groothaert, M. A. Vance, B. F. Sels, R. A. Schoonheydt, E. I. Solomon, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18908.

[13] R. A. Himes, K. D. Karlin, *Curr. Opin. Chem. Biol.* **2009**, *13*, 119.

[14] E. A. Lewis, W. B. Tolman, *Chem. Rev.* **2004**, *104*, 1047.

[15] I. Sanyal, M. Mahroof-Tahir, S. Nasir, P. Ghosh, B. I. Cohen, Y. Gultneh, R. Cruse, A. Farooq, K. D. Karlin, S. Liu, J. Zubieta, *Inorg. Chem.* **1992**, *31*, 4322.

